DEVELOPMENT AND VALIDATION OF NEW ANALYTICAL METHODS FOR THE ESTIMATION OF LACOSAMIDE BY UV SPECTROSCOPY, RP-HPLC AND HPTLC METHOD

Dissertation Submitted to

The Tamil Nadu Dr. M.G.R. Medical University

Chennai - 600032.

In partial fulfillment for the award of Degree of

MASTER OF PHARMACY

(Pharmaceutical Analysis)

Submitted by

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MELMARUVATHUR – 603319

MAY 2012

CERTIFICATE

This is to certify that the research work entitled "**DEVELOPMENT AND VALIDATION OF NEW ANALYTICAL METHODS FOR THE ESTIMATION OF LACOSAMIDE BY UV SPECTROSCOPY, RP-HPLC AND HPTLC METHOD**" submitted to The Tamil Nadu Dr. M.G.R Medical University in partial fulfillment for the award of the Degree of Master of Pharmacy (Pharmaceutical Analysis) was carried out by **R.E.SEENUVASAN** (Reg No. **26106130**) in the Department of Pharmaceutical Analysis under my direct guidance and supervision during the academic year 2011-2012.

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ACKNOWLEDGEMENT

First and foremost, I wish to express my deep sense of gratitude to his Holiness**ARULTHIRU AMMA** for his ever growing Blessings in each step of the study. I am grateful to **THIRUMATHI LAKSHMI BANGARU ADIGALAR,** Vice President, ACMEC Trust, Melmaruvathur for given me an opportunity and encouragement for the way in completing the study.

With great respect and honor, I extend my thanks to our Managing Director **MR. G. B. ANBALAGAN,** Managing Trustee, Adhiparasakthi Hospital and Research Institute, Melmaruvathur for his excellence in providing skillful and compassionate spirit of unstinted support to our department for carrying out research work entitled **"DEVELOPMENT AND VALIDATION OF NEW ANALYTICAL METHODS FOR THE ESTIMATION OF LACOSAMIDE BY DERIVATIVE SPECTROSCOPY, RP-HPLC AND HPTLC METHOD"**.

On a personal note I wish to avail my deep sense of thanks to **Prof. (Dr.) T.VETRICHELVAN, M. Pharm., Ph.D.** Principal, Adhiparasakthi College of Pharmacy, for all the support offered by him during the period of my entire dissertation work**,** I wish to sincerely record my deepest gratitude and thanks for his kind cooperation and initiative guidance, constructive criticism with enthusiastic encouragement which enabling me to complete this project work successfully.

I have great pleasure in express my sincere heartfelt thanks to **Mrs. (Dr.) D. NAGAVALLI, M. Pharm.**, **Ph.D.,** Professor, **Mr. K. ANANDA KUMAR, M. Pharm.,** Associate Professor**,** and **Mrs. G. ABIRAMI, M. Pharm.,** Assistant Professor**,** and **Mr. J. SAMINATHAN, M. Pharm.,** Assistant Professor Department of

Pharmaceutical Analysis for encouragement and support for the successful completion of this work.

My sincere thanks to our lab technicians **Mr. M. GOMATHI SHANKAR, D. Pharm.,** and **Mrs. S. KARPAGAVALLI, D. Pharm.,** for their kind help throughout this work.

I am indeed very much thankful to the librarian **Mr. M. SURESH, M.L.I.S.,** for providing all reference and literature for the completion of this project.

My sincere thanks to **Mrs. PRATHIMA MATHUR**, Managing Director, Pharma Information Centre, Chennai for helping in the literature collection.

My indepted thanks and appreciation goes to dear friend. **Mr**. **R. SABARINATHAN**, **Mr. R. LOGESH, Mr. G. VAMSI KRISHNA, Mr. D. MANI SAI, Mr. P. KISHORE Mr. C. KUMAR** and my classmate for their non forgettable help, moral support, inspiration and kind co-operation constant encouragement and constructive criticism during the course of my work.

A special word of thanks to my ever loving **College** S**taffs, Friends, Seniors and Juniors** for their kind moral support during my work

Finally yet importantly, I gratefully forward my affectionate thanks to my Mother **Mrs. E. SELVI**, and my father **Mr. R. ELUMALAI**, and my brother **Mr. E. SIVA** and my sisters **Mrs. E. SANKARI, Mrs. E. JEEVA** for their frequent prayers, which has sustained me a lot in the successful completion of my project work**.**

R.E.SEENUVASAN

Dedicated To

My Parents &

All My Friends...✍

CONTENTS

LIST OF FIGURES

15.	CALIBRATION CURVE OF LACOSAMIDE BY RP-HPLC
16.	CHROMATOGRAM FOR ANALYSIS OF FORMULATION – (LACOSAM) REPEATABILITY – 1
17.	CHROMATOGRAM FOR ANALYSIS OF FORMULATION – (LACOSAM) REPEATABILITY – 2
18.	CHROMATOGRAM FOR ANALYSIS OF FORMULATION – (LACOSAM) REPEATABILITY – 3
19.	CHROMATOGRAM FOR ANALYSIS OF FORMULATION – (LACOSAM) REPEATABILITY – 4
20.	CHROMATOGRAM FOR ANALYSIS OF FORMULATION – (LACOSAM) REPEATABILITY – 5
21.	CHROMATOGRAM FOR ANALYSIS OF FORMULATION – (LACOSAM) REPEATABILITY – 6
22.	CHROMATOGRAM FOR ANALYSIS OF FORMULATION – (LACOSAM) REPEATABILITY – 7
23.	CHROMATOGRAM FOR ANALYSIS OF FORMULATION – (LACOSAM) REPEATABILITY – 8
24.	CHROMATOGRAM FOR ANALYSIS OF FORMULATION – (LACOSAM) REPEATABILITY – 9
25.	CHROMATOGRAM FOR FIRST RECOVERY ANALYSIS OF FORMULATION (LACOSAM)
26.	CHROMATOGRAM FOR SECOND RECOVERY ANALYSIS OF FORMULATION (LACOSAM)
27.	CHROMATOGRAM OF THIRD RECOVERY ANALYSIS OF FORMULATION (LACOSAM)
28.	SELECTION OF DETECTION WAVELENGTH FOR HPTLC METHOD
29.	LINEARITY CHROMATOGRAM OF LACOSAMIDE BY HPTLC [1 µg/ mL]
30.	LINEARITY OF CHROMATOGRAM OF LACOSAMIDE BY HPTLC [2 µg/ mL]
31.	LINEARITY OF CHROMATOGRAM OF LACOSAMIDE BY HPTLC [3 µg/ mL]

32.	LINEARITY OF CHROMATOGRAM OF LACOSAMIDE BY HPTLC [4 µg/ mL]
33.	LINEARITY OF CHROMATOGRAM OF LACOSAMIDE BY HPTLC [5 µg/ mL]
34.	LINEARITY OF CHROMATOGRAM OF LACOSAMIDE BY HPTLC [6µg/ mL]
35.	CALIBRATION CURVE OF LACOSAMIDE BY HPTLC
36.	CHROMATOGRAM FOR THE ANALYSIS OF FORMULATION – (LACOSAM) REPEATABILITY – 1
37.	CHROMATOGRAM FOR THE ANALYSIS OF FORMULATION – (LACOSAM) REPEATABILITY – 2
38.	CHROMATOGRAM FOR THE ANALYSIS OF FORMULATION – (LACOSAM) REPEATABILITY – 3
39.	CHROMATOGRAM FOR THE ANALYSIS OF FORMULATION – (LACOSAM) REPEATABILITY – 4
40.	CHROMATOGRAM FOR THE ANALYSIS OF FORMULATION – (LACOSAM) REPEATABILITY – 5
41.	CHROMATOGRAM FOR THE ANALYSIS OF FORMULATION – (LACOSAM) REPEATABILITY – 6
42.	CHROMATOGRAM FOR FIRST RECOVERY ANALYSIS OF FORMULATION (LACOSAM) BY HPTLC
43.	CHROMATOGRAM FOR SECOND RECOVERY ANALYSIS OF FORMULATION (LACOSAM) BY HPTLC
44.	CHROMATOGRAM FOR THIRD RECOVERY ANALYSIS OF FORMULATION (LACOSAM) BY HPTLC

LIST OF TABLES

LIST OF ABBREVIATIONS USED

IR	-	Infra red
ICH	-	International Conference on Harmonization
λ	-	Lambda
LOD	-	Limit of Detection
LOQ	-	Limit of Quantification
µg/ mL	-	Microgram per Milliliter
mg/ tab	-	Milligram Per tablet
MW	-	Molecular weight
mL	-	Millilitre
mM	-	Milli Mole
nm	-	Nanometer
pH	-	Negative Logarithm of Hydrogen Ion
%	-	Percentage
% RSD	-	Percentage Relative Standard Deviation
Rt or t_R	-	Retention Time
S.D.	-	Standard Deviation
S.E.	-	Standard Error
UV	-	Ultraviolet
USP	-	United States Pharmacopoeia
HPLC	-	High Performance Liquid Chromatography
HPTLC	-	High Performance Thin Layer Chromatography
°C	-	Degree Celsius
Gms	-	Grams
µl	-	Microlitre
rpm	-	Rotations Per Minute
µ	-	Micron
v/v	-	Volume/Volume
LAC	-	Lacosamide
R_F	-	Retardation factor

INTRODUCTION

1. INTRODUCTION

1.1 Introduction to Analytical chemistry

(P C Kamboj, 2003. Annees A. Siddiqui, 2006*)*

The pharmaceutical analysis defined as "the branch of practical chemistry which deals with the resolution, separation, identification, determination and purification of a given sample of a medicine, the detection and estimation of impurities, which may be present in drug substance (or) given sample of medicine". The substance may be a single compound or a mixture of compounds and may be in the form a tablet, pill, capsule, ampoule, liquid, mixture or an ointment.

The quality control tests involve methods which embrace chemicals, physio - chemical/ instrumental, microbiological (or) biological procedures.

The pharmaceutical analysis deals with the subject of determining the composition of material in terms of the elements or compound (drug) present in the system.

Any type of analysis involves two steps

Identification (qualitative)

Estimation (quantitative)

In qualitative analysis, a reaction is performed in such a way as to indicate the formation of a precipitate, a change of a colour , the dissolution of a precipitate/ complex formation and the evaluation of a gas.

Quantitative analysis is performed ordinarily through five steps. They are sampling, dissolution, precipitation, measurement and calculation.

Method of assay

It indicates the quantitative determination of principal ingredients of the official substances and in preparations.

Qualitative analysis

This is practiced in order to establish the composition of a naturally occurring or artificially synthesized/ manufactured substance.

Qualitative analysis

1. **Chemical Methods**
   a) Titrimetric analysis
   b) Gravimetric analysis
   c) Gasometric analysis
2. **Physio - Chemical Methods (Instrumental Methods)**
3. **Microbiological Procedures**
4. **Biological Procedures**

I. Chemical Methods

a. Titrimetric Analysis

The analysis based on the fact that in all balanced chemical reactions utilized for the purpose. Equivalent weight of one substance reacts quantitatively with the equivalent weight of the other substance. The difference types of titration are as follows

Acid base titrations (neutralization titrations)

Non- aqueous titrations

Redox titrations (redox = oxidation - reduction)

Precipitation titrations

Complexometric titrations

b. Gravimetric Analysis

This method involves the conversion of the element or a radical to be determined into a pure stable compound readily convertible into a form suitable for weighing.

c. Gasometric Analysis

This type of analysis involves the measurement of the volume of gases. The volume of a gas set free in a given chemical reaction under the conditions similar to those described in the process. It may be noted that the volume of gas is taken at normal conditions and pressure or standard temperature and pressure (NTP/ STP) which is a temperature of 0^0C (273.09^o K) and the pressure of a column of 760mm/ Hg at 0^0C. If the reaction is taken place under different temperature and pressure the volume is adjusted to standard conditions. A decrease in the volume of gas when a suitable reagent is placed to absorb one of the gases present. This decrease in volume is also reduced to STP.

The gases cyclopropane, CO_2, NO_2, oxygen, octyl nitrite, Nitrogen, amyl nitrite, ethylene and helium are determined by gasometric analysis. The measurement of volume of gases is usually done by means of gas burettes or nitrometers.

II. Physio - Chemical Methods (Instrumental Methods)

Initially analytical methods were depending on extraction procedure, volumetric and gravimetric methods. All these methods are nearly replaced by advanced instrumental methods. These methods are more sensitive, specific and accurate but cost factors of the instruments and their maintenance are the main draw backs. Various instrumental methods are classified depending on the property analyzed.

Shows different instrumental methods with basic principle

Sr. N0.	MEHTOD	BASIC PRINCIPLE
A	ELECTROANALYTICAL METHODS	
1	Potentiometry	Concerned with change in electrical properties of the system measures the change in electrode potential during a chemical reaction of the system
2	Conductometry	Measures the change in electrical conductivity during a chemical reaction
3	Polarography	Measure the current at various applied potential indicating the polarization at indicator electrode
4	Amperometry	Measure the change (or decrease) in current at a fixed potential during addition of titrant
B	SPECTROSCOPIC METHODS	
1	Absorption Spectroscopy (Ultraviolet-Visible and Infrared)	Measure the absorbance or percent transmittance during the interaction of monochromatic radiation (or particular wavelength) by the same
2	Fluorimetry	Measure the intensity of fluorescence caused by emission of electromagnetic radiation due to absorption of UV radiation
3	Flame Photometry	Measure the intensity of emitted light of particular wave length emitted by particular element
4	Turbidimetry	Measure the turbidity of a system by passing light beam in a turbid media
5	Nephlometry	Measure the opalescence of the medium by reflection of light by a colloidal solution
6	Atomic Absorption	Measure the intensity of absorption when

	Spectrometry	atoms absorbs the monochromatic radiation
7	X-Ray Spectroscopy	Measure the position and intensity of spectral lines during emission of X ray spectrum by atoms under influence of X rays
8	Refractometry	Measure the refractive index by causing refraction of light by matter
9	Polarimetry	Measure optical reaction by causing the rotation of plane polarized light
C	Mass Spectroscopy	Observe the position and intensity of signals in mass spectrum by causing the ionization of molecules
D	NMR Spectroscopy	Observe the position and intensity lines in NMR spectrum when proton interact with electromagnetic radiation in radio frequency region
E	Thermal Methods	Measure the physical parameters of the system as a function of temperature. It includes thermo gravimetry, derivative gravimetry, differential thermal analysis
F	Radiometric Methods	Measure the radioactivity either present naturally or induced artificially

III. Microbiological Methods

`In a microbiological assay, a comparison of inhibition of the growth of bacteria by a measured concentration of the antibiotic, which is to be examined, is made with that produced by known concentration of the standard preparation of an antibiotic having known activity.

IV. Biological Methods

When the potency of a drug or its derivative cannot be properly determined by physical or chemical methods and where it is possible to observe the biological effects of the drug on some type of living matter. The biological assays are carried out. The basis of such assay is to determine how much of the sample gives the same biological effect as a given quantity of the standard preparation. The sample and standard preparation are tested under identical conditions in all respect. In a typical bio – assay, a stimulus is applied to a subject is referred to as the dose and is indicated by a weight or in terms of the concentration of the preparation. The application of stimulus on a subject produces some observable effect and this is called the response. The response may be measured by the total weight or weight of some organ of the subject, blood sugar concentration, diameter of inhibition zone or by some other physiological symptoms.

1.2.1 ULTRAVIOLET SPECTROSCOPY (Beckett and Stenlake, 2002)

This technique of ultra violet spectroscopy is one of most frequently employed method in pharmaceutical analysis. It involves the measurement of the amount of UV radiation (190 - 380 nm) or visible (380 - 800 nm) radiation absorbed by a substance in solution. Ultraviolet spectroscopy involves the promotion of electrons (σ, π, n electrons) from the ground state to higher energy state. It is useful to measure the number of conjugated double bonds and also aromatic conjugation with the various molecules.

The ultraviolet region of the electromagnetic spectrum is frequently subdivided into as follows:

- Far vacuum Ultraviolet region (10 - 200 nm)
- Near ultraviolet region (200 - 400 nm)

- Visible region (380 - 780 nm)

Origin and theory of ultraviolet spectra (Gurdeep R. Chatwal, et al., 2000**)**

Ultraviolet absorption spectra arise from transition of electron (or) electrons with in a molecule or an ion from a lower to a higher electron energy level and the ultraviolet emission spectra arise from the reverse type of transmission. For radiation to cause electronic excitation it must be in the UV region of the electromagnetic spectrum.

Energy absorbed in the ultraviolet region produces change in the electronic energy of the molecule resulting from transition of valence electrons in the molecule. Three distinct types of electrons are involved in organic molecule. These are as follows

σ – Electrons

Theses electrons are involved in saturated bonds, such as those between carbons and hydrogen in paraffins. These bonds are known as σ bonds. As the amount of energy required to excite electron in σ bonds is much more than that produced by UV light, compounds containing σ bonds do not absorb UV radiation. These electrons do not absorb near UV radiation but absorb at vacuum UV radiation.

π – Electrons

These electrons are involved in unsaturated hydrocarbons. Typical compounds with π bonds are trienes and aromatic compounds.

n– Electrons

These electrons are non bonded electrons which are not involved in any bonding between atoms in molecules. (eg.) S, O, N and halogens.

Types of Electronic Transitions (Y.R. Sharma, 2009*)*

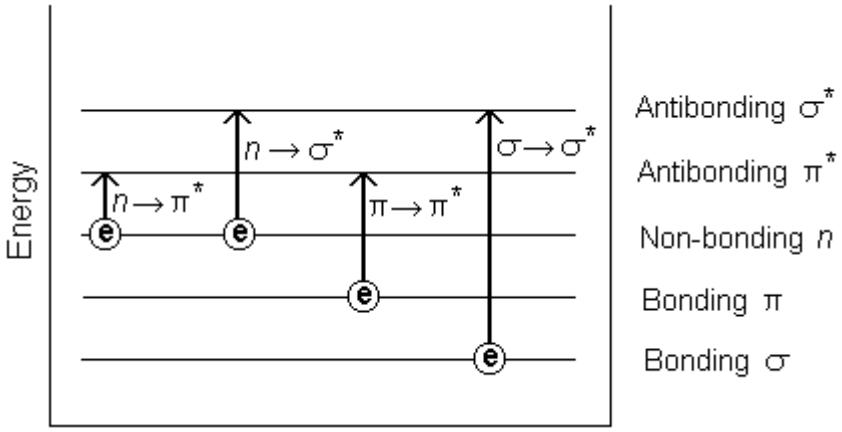
A molecule is excited by the absorption of energy (UV or Visible light). Its electrons are promoted from a bonding to an anti bonding orbital.

i. The anti bonding orbital which is associated with the excitation of σ – electrons is called σ* anti bonding orbital. So σ to σ* transition take place when σ (sigma) electron is promoted to antibody (σ) orbital. It is represented as σ ⟶ σ* transition.

ii. When a non - bonding electron gets promoted to an antibody sigma orbital (σ*) then it represents n - σ* transition.

iii. Similarly π ⟶ π* transition represents the promotion of π electrons to an anti bonding π orbital π* orbital.

iv. When an n- electron (non- bonding) is promoted to anti bonding π orbital. It represents n ⟶ π* transition.



Electronic excitation energies

a) σ ⟶ σ* transition

The organic compounds in which all the valence shell electrons are involved in the formation of sigma bonds do not show absorption in the normal ultraviolet region, but occur in vacuum UV region (125 - 135).

e.g. methane, ethane, propane, cyclopropane.

b) n ⟶ σ* transition

This type of transition takes place in saturated compounds containing one hetero atom with unshared pair of electrons (n electrons).

e.g. alcohol, ethers, aldehydes, ketones, amines etc.,

Such transition require comparatively less energy than that required for σ ⟶ σ* transitions.

c) π ⟶ π* transition

This type of transition occurs in the unsaturated centers of the molecule in compounds containing double or triple bonds and also in aromatics. The excitation of π electrons smaller energy and hence transition of this type occurs at longer wavelength.

e.g. alkenes, alkynes, carbonyl compounds, cyanides.

d) n ⟶ π* transition

In this type of transition an electron of unshared electron pair on hetero atom gets excited to π* anti body orbital. This type of transition requires least amount of energy out of all the transitions..

The absorption laws (Y.R. Sharma, 2009)

There are two laws which govern the absorption of light by the molecules. These are,

(1) Lambert's Law

(2) Beer's Law

Lambert's Law

When a beam of monochromatic radiation passes through a homogenous absorbing medium, the rate of decrease of intensity of radiation with thickness of absorbing medium is proportional to the intensity of incident radiation.

$$I = I_0 e^{-kt}$$

Where, I_0 = Intensity of incident light

I = Intensity of emerged light

t = Thickness of the medium

Beer's Law

When a beam of monochromatic radiation is passed through a solution of an absorbing substance, the rate of decrease of intensity of radiation with concentration of the absorbing solution is directly proportional to the intensity of incident radiation.

$$I = I_0 e^{-kc}$$

Where, I_0 = intensity of incident light

I = Intensity of emerged light

c = concentration of the absorbing species

From these laws, the following empirical expression of Beer - Lambert's Law was constructed

$$\text{Log } (I_0/I_T) = Ɛct = A$$

Where, A= Absorbance or optical density or extinction co-efficient

Ɛ = Molecular extinction co-efficient

c = Concentration of drug

t = Path length

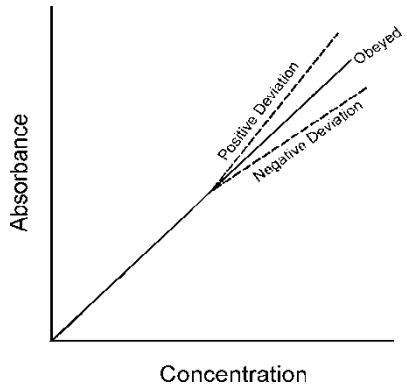
Limitations of Beer Lambert's Law

1. When different forms of the absorbing molecules are in equilibrium as in keto-enol tautomers.
2. When fluorescence compounds are present.
3. When solute and solvent forms complex through some sorts of association.

Deviations from Beer's Law (Gurdeep R. Chatwal, et al., 2000*)*

According to Beer's law, a straight line passing through the origin should be obtained, when a graph is plotted between absorbance and concentration. But there is always a deviation from linear relationship between absorbance and concentration and

intact the shape of an absorption curve usually changes with changes in concentration of solution and unless precautions are observed. Deviations from the law may be positive or negative according to whether the resulting curve is concave upward or concave downward.



The latter two are generally known as instrumental deviation and chemical deviation.

Instrumental deviations

Stray radiation, improper slit width, fluctuation in single beam.

Chemical deviations

Hydrolysis, Association, Polymerization, Ionization, Hydrogen bonding.

Deviations from Beer's Law can arise due to the following factors

1. Beer's law will hold over a wide range of concentration provided the structure of coloured ion or of the coloured non electrolyte in the dissolved state does not change with concentration. If a coloured solution is having a foreign substance whose ions do not react chemically with the coloured components, its small concentration does not affect the light absorption and may also alter the value of extinction co - efficient.
2. Deviations may also occur if the coloured solute ions dissociates or associates.
3. Deviations may also occur due to the presence of impurities that fluorescence or absorb at absorption wavelength.

4. Deviations may occur if monochromatic light is not used.
5. Deviations may occur if the width of slit is not proper and therefore it allows undesirable radiations to fall on the detector.
6. Deviations may occur if the solution undergoes polymerization.
7. Beer's law cannot applied to suspensions but the latter can estimated calorimetrically after preparing a reference curve with known concentrations.

Choice of solvent *(*Gurdeep R. Chatwal, et al., 2000*)*

A suitable solvent for ultraviolet spectroscopy should meet the following requirements.

(i) It should not itself absorb radiations in the region under investigation.

(ii) It should be less polar so that it has minimum interaction with the solute molecule.

The most commonly employed solvent is 95% ethanol. It is cheap, has good dissolving power and does not absorb radiation above 210 nm. In other words it is transparent above 210 nm. Commercial ethanol should not be used as it contains some benzene which undergoes absorption in the UV range at about 280.Some other solvents which are transparent above 210 nm are n-hexane, cyclohexane, methanol, water and ether, Benzene, chloroform, and carbon tetrachloride cannot be used because they absorb in the range of about 240 - 280 nm.Hexane and other hydrocarbon are sometimes preferred to polar solvents because they have minimum interaction with the solute molecules.

Solvent Effects

The position and the intensity of absorption maximum is shifted for a particular chromophore by changing the polarity of the solvent. By increasing the polarity of solvent, compounds like dienes and conjugated hydrocarbons do not experience any

appreciable shift. The absorption maximum for the polar compounds is usually shifted with the change in polarity of the solvents. α and β unsaturated carbonyl compounds show two different shifts.

a) n → π* transition

The absorption band moves to short wavelength by increasing the polarity of the solvent. In n → π* transition the ground state is more polar as compared to the excited state. The hydrogen bonding with solvent molecules take place to lesser extent with the carbonyl group in the excited state.

b) π → π* transition

The absorption band moves to longer wavelength by increasing the polarity of the solvent. The dipole interactions with the solvent molecules lower the energy of the excited state more than that of the ground state.

CHROMOPHORES AND AUXOCHROMES *(*Jagmohan, 2005)

A. Chromophores

"The presence of one or more unsaturated groups responsible for electronic absorption is called as chromophores".

e.g. C = C, C ≡ C, C = N, C ≡ N, C = O

B. Auxochromes

An Auxochrome is an auxiliary group which interacts with the chromophore causing a bathochromic shift. The presence of Auxochrome causes a shift in the UV or Visible absorption maximum to the longer wavelength.

e.g. NH_2, NR_2, OH, OR, SH, NHR.

INSTRUMENTATION (Gurdeep R. Chatwal, et al., 2000*)*

All photometers, colorimeters and spectrophotometers have the following basic components

1) **Radiation Source**

i) It must be stable.

ii) It must be of sufficient intensity for the transmitted energy to be detected at the end of the optical path.

iii) It must supply continuous radiation over the entire wavelength region in which it is used.

UV region

Tungstan lamp, Hydrogen discharge lamp, Deuterium discharge lamp, Xenon arc Lamp.

2) Filters and monochromatoros

The filters and monochromators are used to disperse the radiation according to the wavelength. The essential of a monochromator are an entrance slit, a dispersing element and an exit slit. The entrance slit sharply defines the incoming beam of heterochromatic radiation. The dispersing element disperses the heterochromatic radiation into its component wavelengths where as exit slit allows the nominal wavelength together with a band of wavelength on either side of it. The position of the dispersing element is always adjusted by rotating it to vary the nominal wavelength passing through the exit slit.

UV region

The dispersing element may be a prism or grating. The prisms are generally made of glass, quartz or fused silica. Glass has the highest resolving power but it is not

transparent to radiation having the wavelength between 2000 and 3000 A because glass absorbs strongly in this region. Quartz and fused silica prism which are transparent throughout the entire UV range are widely used in UV spectrophotometers.

Types of monochromators

1) Prisms

2) Gratings

1) Prisms are of two types

i) Refractive prism

ii) Reflective prism

2) Gratings are of two types

i) Diffraction grating

ii) Transmission grating

Visible region

Filters or monochromators or both are used

Filters

A light filter is a device that allows light of the required wavelength to pass but absorbs light of other wavelengths wholly or partially. Thus, a suitable filter can be select a desired wavelength band. It means that a particular filter may be used for a specific analysis. If analysis is carried out for several species, a large number of filters have to be used and interchanged. This method is very useful for routine analysis.

Types of filters

Filters are two types,

i) Absorption filters

ii) Interference filters

3) Sample cell

These are containers for the sample and reference solutions and must be transparent to the radiation passing through.

UV region

The cells made up of quartz.

Visible region

The cells made of glass.

4) Detectors

Detectors used in UV/Visible spectrophotometers can be called as photometric detectors. In these detectors the light energy is converted to electrical signal which can be recorded. The types of detectors used are

Barrier Layer cell (or) Photo Voltaic cell.

Photo tubes (or) Photo emissive tubes

Photomultiplier tubes

Photo diode

QUANTITATIVE SPECTROPHOTOMETRIC METHODS (Beckett and Stenlake, 2002*)*

The assay of an absorbing substance may be quickly carried out by preparing a solution in a transparent solvent and measuring its absorbance at a suitable wavelength. The wavelength normally selected is a wavelength of maximum absorption (λ_{max}), where small errors in setting the wavelength scale have little effects on the measured absorbance.

a. Assay of substances in single component samples

Absorption spectroscopy is one of the most useful tools available to the chemist for quantitative analysis. The most important characteristics of photometer and spectrophotometric method are high selectivity and ease of convenience. Quantitative analysis (assay of an absorbing substance) can be done using following methods.

- Use of $A_{1\,cm}^{1\%}$ values
- Use of calibration graph (multiple standard method)
- By single or double point standardization method.

i) Use of $A_{1\,cm}^{1\%}$ values

This method can be used for estimation of drug from formulations or raw material, when reference standard not available. The use of standard value $A_{1\,cm}^{1\%}$ avoids the need to prepare a standard solution of the reference substance in order to determine its absorptivity, and is of advantage in situations where it is difficult or expensive to obtain a sample of the reference substance.

ii. Use of calibration graph

In this procedure the absorbances of a number (typically 4-6) of standard solutions of the reference substance at concentrations encompassing the sample concentrations are measured and a calibration graph is constructed. The concentration of the analyte in the sample solution is read from the graph as the concentration corresponding to the absorbance of the solution. Calibration data are essential if the absorbance has a non-linear relationship with concentration, or if the absorbance or linearity is dependent on the assay conditions. In certain visible spectrophotometric assays of colourless substances, based upon conversion to coloured derivatives by heating the substance with one or more reagents, slight variation of assay conditions,

e.g. P^H, temperature and time of heating, may rise to a significant variation of absorbance, and experimentally derived calibration data are required for each set of samples.

iii. Single point standardization

The single point procedure involves the measurement of the absorbance of a sample solution and of a standard solution of the reference substance. The standard and the sample solution are prepared in similar manner; ideally the concentration of the standard solution should be close to that of the sample solution. The concentration of the substance in the sample is calculated using following formula.

$$\mathbf{C_{test} = A_{test} \times C_{std} / A_{std}}$$

Where,

C_{test} and C_{std} are the concentration in the sample and standard solutions respectively.

A_{test} and A_{std} are the absorbance of the sample and standard solutions respectively

b. Assay of substances in multi component samples

The spectrophotometric assay of drugs rarely involves the measurement of absorbance of samples containing only one absorbing component. The pharmaceutical analyst frequently encounters the situation where the concentration of one or more substances is required in samples known to contain other absorbing substances which potentially interfere in the assay. Unwanted absorption from these sources is termed irrelevant absorption and if not removed, imparts systematic errors to the assay of the drug in the sample. A number of modifications to the simple spectrophotometric procedure for single-component samples are available to the analyst, which may

eliminate certain sources of interferences and permit the accurate determination of one or all of the absorbing components.

The basis of all the spectrophotometric technique for multicomponent samples is the property that at all wavelengths:

a) The absorbance of a solution is the sum of absorbances of the individual components.

b) The measured absorbance is the difference between the total absorbance of the solution in the sample cell and that of the solution in the reference (blank) cell.

The determination of the multi-component samples can be done by using the following methods,

- Simultaneous equation method
- Absorbance ratio method
- Geometric correction method
- Orthogonal polynomial method
- Difference spectrophotometry
- Derivative spectrophotometry
- Chemical derivatization

1.2.2 Derivative Spectrophotometry

Derivative spectrophotometry involves the conversion of a normal spectrum to its first, second or higher derivative spectrum. The transformations that occur in the derivative spectra are understood by reference to a Gaussian band which represents an ideal absorption band. In the context of derivative spectrophotometry, the normal absorption spectrum is referred to as the fundamental, zeroth order or D° spectrum.

The first derivative (D^1) spectrum is a plot of the ratio of change of absorbance with wavelength against wavelength, i.e. a plot of the slope of the fundamental spectrum against wavelength or a plot of dA/dλ Vs λ. At λ2 and λ4, the maximum positive and maximum negative slope respectively in the D°. Spectrums correspond with maximum and a minimum respectively in the D^1 spectrum. The λ_{max} at λ_3 is a wavelength of zero slopes and gives dA/d λ 0, i.e. a cross-over point, in the D1 spectrum.

The second derivative (D^2) spectrum is a plot of the curvature of the D° spectrum against wavelength or a plot of $d^2A/d\lambda^2$Vs λ. The maximum negative curvature at λ_3 in the D° spectrum gives a minimum in the D^2 spectrum, and at λ_1 and λ_5 the maximum positive curvature in the D° spectrum gives two small maxima called 'satellite' bands in the D^2 spectrum. At λ_2 and λ_4 the

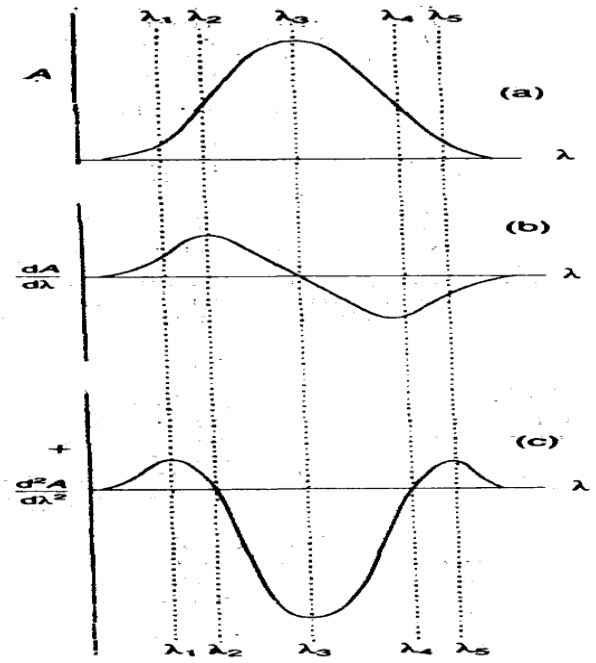


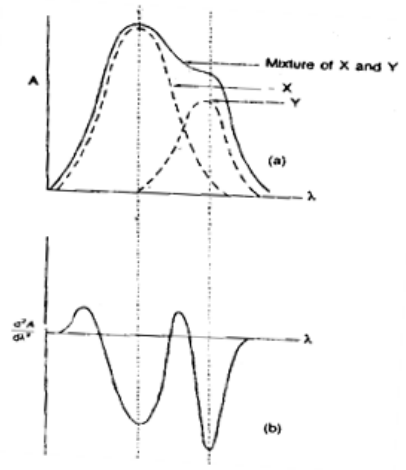
Figure. The zeroth (a), first (b) and second (c) derivative spectra of a Gaussian band.Wavelengths of maximum slope and zero curvature in the D° spectrum correspond with cross-over points in the D^2 spectrum.

In summary, the first derivative spectrum of an absorption band is characterized by a maximum, a minimum, and a cross-over point at the λ_{max} of the absorption band. The-second derivative spectrum is characterized by two satellite maxima and an inverted band of which the minimum corresponds to the λ_{max} of the fundamental band.

These spectral transformations confer two principal advantages on derivative spectrophotometry. Firstly, an even order spectrum is of narrower spectral bandwidth than its fundamental spectrum. Secondly, derivative spectrophotometry discriminates in favour of substances of narrow spectral bandwidth against broad bandwidth substances. This is because 'the derivative amplitude (D), i.e. the distance from a maximum to a minimum, is inversely proportional to the fundamental spectral bandwidth (14') raised to the power (n) of the derivative order

. Thus, $\mathbf{D \alpha (1/W)^n}$

Consequently, substances of narrow spectral bandwidth display larger derivative amplitudes than those of broad bandwidth substance



(a) The individual spectra of two components X and Y in admixture and their combined spectrum. (b) The second derivative spectrum of the mixture showing improved resolution of the individual bands.

The enhanced resolution and bandwidth discrimination increases with increasing derivative order. However, it is also found that the concomitant increase in electronic noise. Inherent in the generation of the higher order spectra, the consequent reduction of the signal-to-noise ratio, place serious practical limitations on the higher order spectra.

1.3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

1.3.1 Introduction to chromatography *(*Gurdeep R. Chatwal, et al., 2000*)*

Chromatography may be defined as a method of separating a mixture of components into individual components through equilibrium distribution between two phases. Chromatographic technique is based on the difference in the rate at which the components of a mixture move through a porous medium (stationary phase) under the influence of some solvent or gas (mobile phase).

The chromatographic method of a separation, in general, involves the following steps

1. Adsorption or retention of a substance or substance on the stationary phase.

2. Separation of the adsorbed substance by the mobile phase.

3. Recovery of the separated substance by a continuous flow of the mobile phase; the method being called elution.

4. Quantitative and qualitative analysis of the eluted substance

1.3.2. Introduction to HPLC **(**B.K. Sharma, 2006*)*

HPLC is a form of liquid chromatography to separate compounds that are dissolved in solution. Compounds are separated by injecting a plug of the sample mixture on to the column. The different compounds in the mixture pass through the column of different

rates due to difference in their partitioning behavior between the mobile liquid phase and the stationary phases.

Advantages:

- Separation is fast and efficient
- Continuous monitoring of the column effluent
- Can be applied to the separation and analysis of very complex mixture
- Accurate quantitative measurements
- Repetitive and reproducible analysis using the same column
- Adsorption, partition, ion exchange and exclusion column separations are excellently made
- Automation of the analytical procedure and data handling
- Both aqueous and non aqueous samples can be analysed
- Providing a high degree of selectivity for specific analysis
- Determination of multiple components in a single analysis

1.3.2.1. Principle of separation in HPLC *(*Willard, 1986*)*

The principle of separation in normal phase and reverse phase mode is the adsorption. When a mixture of components is introduced in to a HPLC column, they travel according to their relative affinities towards the stationary phase. The component which has more affinity towards the adsorbent travels slower. The components which have less affinity towards the stationary phase travel faster. Since no two components have the same affinity towards the stationary phase the components are separated.

Types of HPLC techniques

a) Based on modes of chromatography
    - Normal phase mode

- Reverse phase mode

b) Based on principle of separation

- Adsorption chromatography
- Ion exchange chromatography
- Ion pair chromatography
- Size exclusion or gel permeation chromatography

- Affinity chromatography
- Chiral phase chromatography

c) Based on elution technique

- Isocratic separation
- Gradient separation

d) Based on scale of operation

- Analytical HPLC
- Preparative HPLC

Normal phase chromatography

In normal phase mode, the stationary phase (silica gel) is polar in nature and the mobile phase is non-polar. In this technique, non-polar compounds travel faster and eluted first. This is because of less affinity between solute and stationary phase. Polar compounds are retained for longer time in the column because of more affinity towards stationary phase and take more time to be eluted from column.

Reverse phase chromatography

In reverse phase technique, a non polar stationary phase is used. The mobile phase is polar is in nature hence polar components get eluted first and non-polar

compounds are retained for a longer time. Since most of the drugs and pharmaceuticals are polar in nature, they are not retained for a longer time and eluted faster, which is advantageous. Different columns used are ODS (octadecyl silane) or C_{18}, C_8 and C_4 etc.

Adsorption chromatography

The principle of separation is adsorption. The separation of components takes place because of the difference in affinity of compounds towards stationary phase.

Ion exchange chromatography

The principle of separation is ion exchange which is reversible exchange of functional groups. In ion exchange chromatography, an ion exchange resin is used to separate a mixture of similar charged ions.

Ion pair chromatography

In ion pair chromatography a reverse phase column is converted temporarily in to ion exchange column by using ion pairing agents like pentane, hexane, heptanes, octane, sulphonic acid sodium salt, tetramethyl or tetraethyl ammonium hydroxide.

Size exclusion or gel permeation chromatography

In this type of chromatography, a mixture of components with different molecular sizes are separated by using gels. The gel used acts as molecular sieve and hence a mixture of substance with different molecular sizes is separated. Soft gels like dextran, agarose or polyacrylamide are used. Semi rigid gels like polystyrene, alkyl dextran in non aqueous medium are also used.

Affinity chromatography

Affinity chromatography uses the affinity of the sample with specific stationary phase. This technique is mostly used in the field of biotechnology, microbiology, biochemistry.

Chiral phase chromatography

Separation of optical isomer can be done by using chiral stationary phases. Different principles operate for different types of stationary phases and for different samples. The stationary phases used for this type of chromatography are mostly chemically bonded silica gel.

Isocratic separation

In this technique, the same mobile phase combination is used throughout the process of separation. The same polarity or elution strength is maintained throughout the process.

Gradient separation

In this technique, a mobile phase combination of lower polarity or elution strength is used followed by gradually increasing the polarity or elution strength.

Analytical HPLC

Where only analysis of the samples are done. Recovery of the samples for re using is normally not done, since the sample used is very low. e.g. µg quantities.

Preparative HPLC

Where the individual fractions of pure compounds can be collected using fraction collector. The collected samples are reused. eg. Separation of few grams of mixtures by HPLC.

HPLC instrumentation

Mobile phase (Mendham, et al., 2002**)**

A successful chromatographic separation depends upon difference in the interaction of the solutes with the mobile phase and stationary phase, and in liquid chromatography the choice and variation of the mobile phase is of critical importance in achieving optimum efficiency. However, before considering the theoretical aspects of solvent choice, here are a few general comments; HPLC grade solvents tend to be costly. To ensure consistent performance, the solvent needs to contain no more than trace amounts of other materials, including water for organic solvents. And if the system uses a UV detector then even small traces of absorbing species will be unacceptable; they must be exhaustively removed. Particulates in the solvent are also highly undesirable, with prolonged use they will lead to wear in the pump and injector and cause blockage of the column.

Although normal laboratory grade solvents could be suitably purified, this is a time consuming step and many laboratories prefer to purchase HPLC grade reagents, including water. HPLC grade reagents can be used directly without further purification, although they may need to degassed immediately before use.

The choice of a suitable mobile phase is vital in HPLC and it is appropriate to refer to the factor influencing this choice. Thus, the eluting power of the mobile phase is determined by its overall polarity, the polarity of the stationary phase and the nature of the sample components. For normal phase separations, eluting power increases with increasing polarity of the solvent, for reverse phase separations, eluting power decrease with increasing solvent polarity.

Solvent Reservoir *(*Ashutoshkar, 2005)

Solvent reservoir comprises of a $1dm^3$ glass bottle having lid and a 1/8 inch diameter ptfc tube to convey the mobile phase from the reservoir to the degassers and then to the pump. As described above, any liquid entering the pump should be free from dust and particulate matter, otherwise these foreign substance may invariably give rise to irregular pumping action, damage seals and valves, irregular behavior of column owing to its contamination, and ultimate blockade of column. Sometimes a stainless steel filter element (of filter size 2 μm) that could be conveniently positioned either in the ptfe - tube in the reservoir or an in - line filter may be employed.

Degassing System

Many liquids dissolve appreciable amounts of atmospheric gases e.g. air or suspended air bubbles that may be a major cause of practical problems in HPLC, specifically affecting the operation of the pump and the detector. However, all such problems may be avoided by degassing the mobile phase by subjecting the mobile phase under vacuum, distillation, spurging with a fine spray of an inert gas of low solubility such as Argon or Helium or by heating and ultra sonic stirring.

Pumping Systems *(*Douglas A. Skoog, 2005*)*

The requirements for liquid chromatographic pumps include

a) Ability to generate pressure of up to 6000 psi (Lb/in^2)
b) Pulse free out put
c) Flow rates ranging from 0.1 to 10 mL/min
d) Flow reproducibilities of 0.5% relative or better
e) Resistance to corrosion by a variety of solvents

Types of pumps

1. Reciprocating piston pump

2. Syringe – type pump
3. Constant pressure pump

Sample injection system (Mendham, et al., 2002**)**

Introduction of the sample is generally achieved is one of two ways, either by using syringe injection or through a sampling valve. Septum injectors allow sample introduction by a high pressure syringe through a self sealing elastomer septum. One of the problem associated with septum injectors is the leaching effect of the mobile phase in contact with the septum, which may give rise to ghost peaks. In general, syringe injection for HPLC is more trouble same than in gas chromatography.

Although the problems associated with septum injectors can be eliminated by using stop flow septum less injection. Currently the most widely used devices in commercial chromatographs are the micro volume sampling valves. Which enable sample to be introduced reproducibly in to pressurised columns without significantly interrupting the flow of the mobile phase. The sample is loaded at atmospheric pressure in to an external loop in the valve and introduced into the mobile phase by an appropriate rotation of the valve. The volume of sample introduced, ranging from 2 µl to over 100 µl, may be varied by changing the volume of the sample loop or by using special variable volumes sample valves. Automatic sample injectors are also available which allow unattended operation of the instrument. Valve injection is preferred for quantitative work because of its higher precision compared to syringe injection.

Columns for HPLC *(*Willard, 1986*)*

Columns are constructed of heavy wall, glass lined metal tubing or stainless steel tubing to withstand high pressures (up to 680 atm) and the chemical action of the mobile phase. The interior of the tubing must be smooth with a very uniform bore diameter. Straight columns are preferred and are operated in the vertical position.

Columns end fitting and connectors must be designed with zero void volume to avoid unswept corners or stagnant pockets of mobile phase that can contribute significantly to extra column band broadening. Packaging is usually retained by inserting stainless steel frits into the end of the column.

Most column lengths range from 10 to 30 cm; short, fast columns are 3 to 8 cm long. For exclusion chromatography, columns are 50 to 100 cm long. The types of columns use in HPLC are,

1. Standard columns
2. Radial compression columns
3. Narrow-Bore columns
4. Short, fast columns
5. Guard columns and In - line filters

Guard column *(*Douglas A. Skoog, 2005*)*

Often, a short guard column is positioned ahead of the analytical column to increase the life of the analytical column by removing particulate matter and contaminants from the solvents. In addition, in liquid-liquid chromatography, the guard column serves to saturate the mobile phase with the stationary phase so that losses of the stationary phase from the analytical column are minimized. The composition of the guard column should be similar to that of the analytical columns, the particle size is usually larger, however, to minimize pressure drop.

Detectors *(*Ashutoshkar, 2005*)*

The main function of the detector in HPLC is to monitor the mobile phase coming out of the column, which in turns emits electrical signals that are directly proportional to the characteristics either of the solute or the mobile phase. The various detectors often used in HPLC may be categorized in to

Bulk property detectors

They specifically measure the difference in some physical property of the solute present in the mobile phase in comparison to the individual mobile phase, for instance

a) Refractive-index detectors

b) Conductivity detectors.

Solute property detectors

They critically respond to a particular physical or chemical characteristic of the solute (in question), which should be ideally and absolutely independent of the mobile phase being used. But complete independence of the mobile phase is hardly to be seen, however signal discrimination is good enough to enable distinctly measurable experimental procedures with solvent changes, such as gradient elution. The solute property detectors include

a) UV- detectors

b) Fluorescence detectors.

Multipurpose detectors

Besides, providing a high degree of sensitivity together with a broad linear response attainable range invariably a particular situation critically demands detectors of more selective nature in the domain at 'analytical chemistry' vis-a-vis 'pharmaceutical analysis' that could be accomplished by using multipurpose detectors, such as "Perkin-Elmer 3D system" that combines UV absorption, fluorescence and conductometric detection.

Electro chemical detectors

Electro chemical detector in HPLC usually refers to either amperometric or coulometric detectors, that specifically measure the current associated with the reduction or oxidation of solutes. As only a narrow spectrum of compounds undergo

electrochemical oxidation, such detectors are quite selective, and this selectively may be further enhanced by monitoring the potential applied to the detector so as to differentiate between various electroactive species. Naturally, electrochemical detection essentially makes use of conducting mobile phase, for instance, inorganic salts or mixtures of water with water miscible organic solvents.

1.4 HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

(Sethi, P.D., 1996, Sethi, P.D., Dilip Chareganokar.1999)

HPTLC is the most simple separation technique today available to the analyst. It can be considered a time machine that can speed your work and allows you to do many things at a time usually not possible with other analytical techniques.

1.4.1 Steps involved in HPTLC

1. Selection of HPTLC plates and sorbent
2. Sample preparation including any clean up and pre – chromatographic derivatization
3. Application of sample
4. Development (separation)
5. Detection including post – chromatographic derivatization
6. Quantitation
7. Documentation

1. Plates

Precoated plates

The pre – coated plates with different support material (Glass, Aluminum and plastic) and with different sorbent layers are available in the different format and thickness by various manufacturers. Usually plates with sorbent thickness of 100 – 250 μm are used for qualitative and quantitative analysis, however for preparative TLC work, plates with sorbent thickness of 1.0 – 2.0 mm are available in addition to

chemically modified layers Aluminum Sheet **(**0.1 mm thick): Aluminum sheet as a support offer the same advantage as polyester support but with increased temperature resistance. However with eluents containing high concentration of mineral acids or concentrated ammonia, one may find problem as they will chemically attack aluminum. Aluminum sheets are otherwise compatible with organic solvents and organic acids such as formic acid and acetic acid. Aluminum Precoated plates in size of 20 x 20 cm are usually procured for economic reasons. These plates can be cut to size and shape (format) to suit particular analysis by using general purpose scissors.

2. Pre – washing of pre – coated plates

Sorbents with large surface area absorb not only water vapours and other impurities from atmosphere but other volatile substances often condense particularly after the packing has been opened and exposed to laboratory atmosphere for a long time. Such impurities including elutable components of the binder usually give dirty zones and fail to give reproducible results. It is only for these reasons that pre-coated plates are always packed with the glass or foil side upward (coated layer downward). To avoid any possible interference due to impurities with the chromatographic separations particularly in case of quantitative work, it is always recommended to clear the plates before actual chromatography. This process is called pre – washing of plates. Excellent results are obtained if the plates are subjected to pre – washing (in cleaning solvent) by continuous mode for some time in a chamber closed by a lid having a slit. After washing, the plates must be dried for a sufficient time to ensure complete removal of the washing liquids (usually for methanol 30 – 60 min at 105° c is required). The washed plates should always be stored in a dust – free atmosphere under ambient conditions. As a result of pre – washing, signal to noise ratio is substantially low and base lines are straighter, which is essential for quantitative analysis by in situ

densitometry. Plates exposed to high humidity or kept on a hand for long time may have to be activated by placing in oven at 110 – 120° c for 30 minutes prior to sample spotting. This step removes water that has been physically absorbed on the surface of the sorbent.

3. Sample preparation and Application

The sample preparation procedure is to dissolve the dosage form with complete recovery of intact compounds(s) of interest and minimum of matrix with a suitable concentration of analyte(s) for direct application on the HPTLC plate. Sample application is the most critical step for obtaining good resolution for quantification by HPTLC. The sample should be completely transferred to the layer, however, under no circumstances, the application process should damage the layer, as damaged layer results in unevenly shaped spots. Wherever possible, use of automatic application devices is recommended for quantitative analysis. Usually 0.5 – 5 μl for HPTLC is recommended keeping the size of starting zone(s) down to minimum of 0.5 – 1 mm in concentration range of 0.1 – 1 μg/mL. substance zones which are too large from the beginning because poor separation as during development spots does tend to become large and more diffused. It is therefore recommended that solution should be applied in small increments with intermediate drying (use cold or hot air or nitrogen in case of labile compounds, asymmetric accelerated evaporation of the solvent from the point of application can lead to local changes in the concentration in spotted substances) particularly when the sample solution is predominantly aqueous.

4. Development (separation)

Mobile phase

Poor grade of solvent used in preparing mobile phases have been found to decrease resolution, spot definition and Rf reproducibility. Mobile phases commonly

called solvent system is traditionally selected by controlled process of trial and error and also based on one's own experience in the field. Use of mobile phase containing more than three or four components should normally be avoided as it is often difficult to get reproducible ratios of different components. The chamber saturation has pronounced influence on the separation profile. When the plate is introduced in to an unsaturated chamber, during the course of development, the solvent evaporates from the plate mainly at the solvent front. Therefore larger quantity of the solvents shall be required for a given distance; hence resulting is increase in Rf values. If the tank is saturated (by lining with filter paper) prior to development, solvent vapors soon get uniformly distributed throughout the chamber as soon as the plate is placed in such a saturated chamber, it soon gets pre loaded with solvent vapors, hence less solvent shall be required to travel a particular distance, resulting in lower Rf values. Time required for saturation will depend on the nature and composition of mobile phase and layer thickness.

Development in a non- saturated or partially saturated atmosphere is recommended with solvents used in a composition leading to phase separation such as mixture of n- butanol, water, glacial acetic acid. However in case of RPTLC, it is always preferable to saturate the chamber with methanol as normally in RPTLC, mobile phase with high water contents are employed. If chromatographic procedure is to be carried out at a controlled relative humidity of the chamber then suitable liquid is placed in one of the troughs of twin-trough chamber. Usually relative humidity of the room is controlled by dehumidifier. However, if experiments are required to be carried at specific relative humidity, then solution of sulphuric acid or salt solutions may be employed. After development the plate is removed from the chamber and mobile phase is removed as completely and as quickly as possible. These steps should preferably be

performed in fume cup board to avoid contamination of laboratory atmosphere. The plates should always be laid horizontally so that while mobile phase evaporates the separated substances will migrate evenly to the surface where they can be easily detected.

Factors influencing separation of components and resolution of spots

- Type of stationary phase – sorbent, particle size, activity
- Type of plates
- Layer thickness
- pH of layer
- Binder for layer
- Melting point
- Solvent purity
- Size of chamber
- Saturation of chamber
- Solvent for sample phase
- Melting point level in chamber
- Size of spot
- Relative humidity
- Temperature
- Flow rate of solvent
- Separation distant
- Mode of development

5. Detection and visualization

One of the most characteristic features of HPTLC is the possibility to utilize post chromatographic off-line derivatization. With the availability of many visualization reagents, findings can be confirmed. These visualization reactions are possible for identification even if the separation is not optimally. As soon as the

development process is complete, the plate is removed from the chamber and dried to remove the mobile phase completely. The zones can be located by various physical, chemical, biological- physiological methods. There is apparently no difficulty in detecting color substances or colorless substances absorbing in short wave UV-region (254 nm) or with intrinsic fluorescence such as riboflavin. The substance which do not have above properties has to be transferred in to detectable substances by means of chromogenic or fluorogenic reagents which are more expensive, time - consuming and complicated. Detection sensitivity depends on the specificity for the reagent employed. Iodine is the universal detection reagent, that detection is usually non-destructive and reversible but certain substances may be altered through non-reversible derivatization such as ethambutol, a totally non UV absorbing compound forms a UV absorbing complex with iodine through charge transfer. Detection under UV light is the first choice and is non-destructive in most of the cases and is commonly employed for densitometric scanning. Derivatization reactions are essentially required for detection when individual compound does not respond to UV or does not have intrinsic fluorescence. It is not significant whether derivatization is pre or post chromatographic however, pre-chromatographic derivatization not only helps in detection but enhances the selectivity of the mobile phase. For post chromatographic derivatization, smaller the chromatographic zone, greater the concentration of the substance leading to increase in detection sensitivity. Other simple detection method is based on wetting and solubility phenomenon. Aluminium oxide, kieselguhr, silica gel or hydrophilic adsorbents. On dipping or spraying the chromatogram with water, lipophillic substances such as steroids, fatty acids, hydrocarbons appear as white (opaque) spot against semi-transparent back ground as such a substances being invisible with water or not wetted. This wetting effect is more prominent if the plate is fully saturated with water and held

against light. The contrast is best immediately after dipping, disappears on drying. Other commonly used reagents are phosphomolybdic acid, antimony trichloride or pentachloride, anisaldehyde - sulphuric acid, dimethyl amino benzaldehyde in sulphuric acid and fluorescein sodium. These reagents yield sufficient stable colored spots for quantitative scanning.

6. Quantitation (evaluation)

Requirements for various steps in HPTLC are more stringent for quantitative analysis. Accurate and precise application of samples is the most critical. Further, the chromatographic development should clearly and completely separate all the compounds of interest with no loss by decomposition, evaporation or irreversible adsorption during application or development. Sample and standard as a rule should as a rule should be chromatographed on the same plate under similar conditions.

Earlier, a typical approach was scrapping the separated analyte zones from support material and extracting with a suitable solvent, compounds thus eluted could be analysed by any convenient analytical method; spectrophotometric, fluorometric or by suitable colour development method. To compensate for interference from the sorbent, usually a blank area of the layer is also eluted simultaneously and used as a blank for final analysis. Such blank's values can be lowered by pre-washing of TLC layer with methanol, methanol-chloroform (1:1) or methylene chloride prior to chromatographic procedure. However, this method of separating and elution has limited application as the compounds under analysis may be irreversibly bound to the HPTLC supporter elution/isolation steps may cause some chemical transformation or there is likelihood of analyte loss during extraction. Layers containing gypsum as binder are considerably

softer and especially suited for preparative chromatography involving scrapping form plate and subsequent elution and estimation.

In situ densitometry

Densitometry is the in situ instrumental measurement of visible, UV absorbance, fluorescence or fluorescence quenching directly on the layer without resorting to scrapping and elution. Since chromatographic zones remit a lower light intensity than the environment around it, absorption spectra can be determined directly on the plate by comparison with substance free area on the sorbent layer. The measurements are usually made by reflection from the plate using single beam, double beam or single beam – dual wavelength operation of scanning instruments. The purpose of the scanner is to convert the spot or band on the layer into chromatogram. The position of the scanned peaks on the recorder chart are related to Rf values of the spots on the layer and peak height or area is related to the concentration of the substance on the spot. The signals which are measured represent the adsorption of transmitted or reflected light that passes through the spot compared to blank portion of the sorbent layer. A calibration curve consisting of scan area of standard versus amount of analyte is constructed and amount of analyte in the sample represented by scan area is interpolated from the standard curve.

Factors influencing in selection of detection wavelength

1. The absorption spectra of compound when recorded in solution or in situ from TLC plate are almost similar. Pre – recorded spectra of these compounds as available in literature may be considered for taking decision.

2. In situ spectra of each component of the formulation may be simultaneously considered for selecting the most suitable wavelength for scanning.

3. Extinction coefficient (E 1% 1 cm) and actual concentration of a compound in the formulation has to be taken into consideration, particularly in respect of compounds with low extinction coefficient.

4. While selecting single wavelength, the interest of minor component in the formulation shall need special consideration.

5. If absorption maxima of individual component of the formulation are quite apart, then the chromatogram must be scanned at individual absorption maxima for obtaining meaningful results.

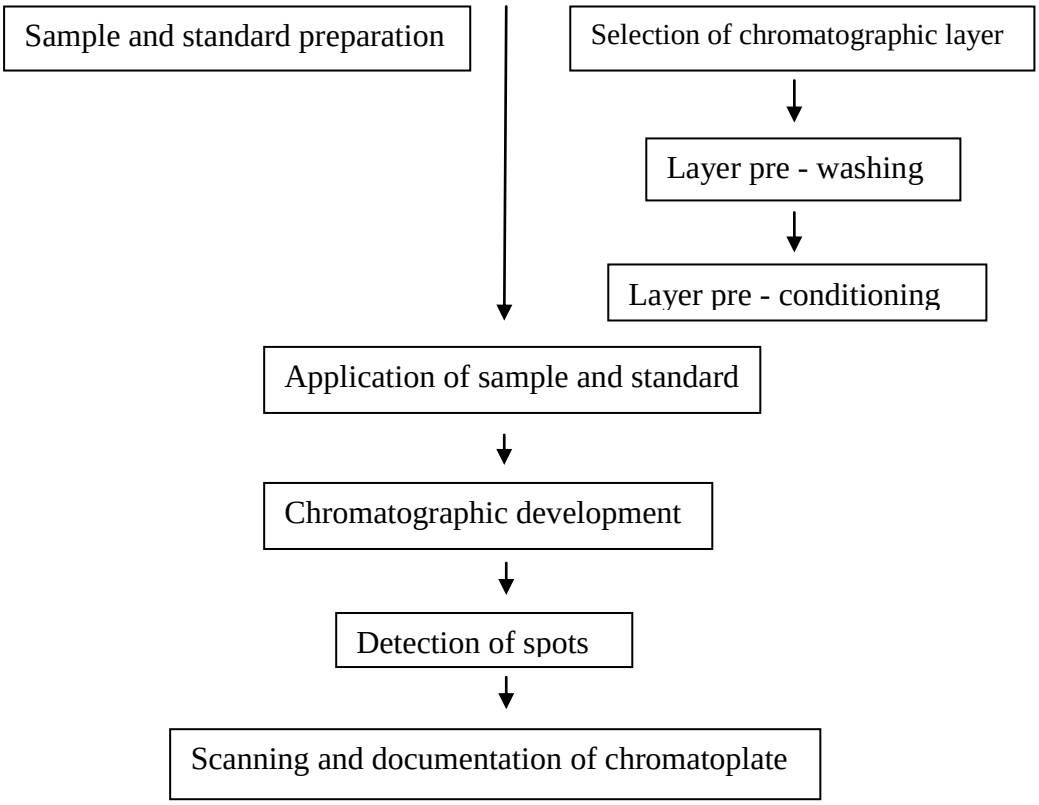
7. Documentation

The use of application scheme and labeling every single chromatogram can avoid mistake in respect of order of application. It is preferable to apply each sample and reference solution twice by following data – pair method. A lead pencil can be used to write on the chromatoplate. The plate should never be marked below the starting point, as the layer is likely to get damaged affecting chromatographic distribution of the substances under analysis, which may ultimately lead to error in scanning. The best way to label the chromatoplate is to mark above the level of solvent point. Immediately after development is completed, the solvent point should be marked with both on left and right hand edges of the plate. To assist the analysts and researchers in practice of HPTLC, E. Merck has recently introduced HPTLC pre - coated plates with an imprinted identification code. The data needed for traceability according to HPTLC such as supplier's name, item number, batch number and individual plate number are imprinted near upper edge of the pre - coated plates. This will not only help in the traceability of analytical data, but will avoid manipulation of data at any stage as coding will automatically get recorded during the photo – documentation.

Stabilization of developed zones

After treatment with the reagent as part of chromatographic derivatization, coloured or fluorescent chromatographic zones are used for quantitative evaluation. It is therefore desirable that the colour or fluorescence thus produced should be stable at least for 30 minutes for carrying out various steps involved in quantitative analysis. There is no general procedure laid down to stabilize the coloured chromatographic zones except to store in atmosphere of nitrogen and protected from light till they are evaluated. However, fluorescent chromatographs can not only be stabilized but often intensified.

A detailed lay out



1.5 ANALYTICAL METHOD VALIDATION

(Code Q2A; Q2B; ICH Guidelines)

Introduction

This document presents a discussion of the characteristics for consideration during the validation of the analytical procedures included as part of registration applications submitted within the EC, Japan and USA. This document does not necessarily seek to cover the testing that may be required for registration in, or export to, other areas of the world. Furthermore, this text presentation serves as a collection of terms, and their definitions, and is not intended to provide direction on how to accomplish validation. These terms and definitions are meant to bridge the differences that often exist between various compendia and regulators of the EC, Japan and USA.

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. A tabular summation of the characteristics applicable to identification, control of impurities and assay procedures is included. Other analytical procedures may be considered in future additions to this document.

Types of Analytical Procedures to be Validated

The discussion of the validation of analytical procedures is directed to the four most common types of analytical procedures:

- Identification tests
- Quantitative tests for impurities content
- Limit tests for the control of impurities
- Quantitative tests of the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product.

Although there are many other analytical procedures, such as dissolution testing for drug products or particle size determination for drug substance, these have not been addressed in the initial text on validation of analytical procedures. Validation of these additional analytical procedures are equally important to those listed herein and may be addressed in subsequent documents.

A brief description of the types of tests considered in this document is provided below.

- Identification tests are intended to ensure the identity of an analyte in a sample. This is normally achieved by comparison of a property of the sample (e.g., spectrum, chromatographic behaviour, chemical reactivity, etc) to that of a reference standard

- Testing for impurities can be either a quantitative test or a limit test for the impurity in a sample. Either test is intended to accurately reflect the purity characteristics of the sample. Different validation characteristics are required for a quantitative test than for a limit test

- Assay procedures are intended to measure the analyte present in a given sample. In the context of this document, the assay represents a quantitative measurement of the major component(s) in the drug substance. For the drug product, similar validation characteristics also apply when assaying for the active or other selected component(s). The same validation characteristics may also apply to assays associated with other analytical procedures (e.g., dissolution).

The objective of the analytical procedure should be clearly understood since this will govern the validation characteristics which need to be evaluated. Typical validation characteristics which should be considered are listed below

Accuracy

Precision

Repeatability

Intermediate Precision

Specificity

Detection Limit

Quantitation Limit

Linearity

Range

Each of these validation characteristics is defined in the attached Glossary. The table lists those validation characteristics regarded as the most important for the validation of different types of analytical procedures. This list should be considered typical for the analytical procedures cited but occasional exceptions should be dealt with on a case-by-case basis. It should be noted that robustness is not listed in the table but should be considered at an appropriate stage in the development of the analytical procedure.

Furthermore revalidation may be necessary in the following circumstances

- changes in the synthesis of the drug substance
- changes in the composition of the finished product
- changes in the analytical procedure.

The degree of revalidation required depends on the nature of the changes. Certain other changes may require validation as well.

TABLE

Type of analytical procedures characteristics	IDENTIFICATION	TESTING FOR IMPURITIES quantitation limit		ASSAY - dissolution (measurement only) - content/potency
Accuracy	-	+	-	+
Precision				
Repeatability	-	+	-	+
Intermediate Precision	-	+ (1)	-	+ (1)
Specificity (2)	+	+	+	+
Detection Limit	-	- (3)	+	-
Quantitation Limit	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+

- signifies that this characteristic is not normally evaluated

+ signifies that this characteristic is normally evaluated

(1) In cases where reproducibility (see glossary) has been performed, intermediate precision is not needed

(2) Lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s)

(3) May be needed in some cases

1.5.1. GLOSSARY

1.5.1.1. ANALYTICAL PROCEDURE

The analytical procedure refers to the way of performing the analysis. It should describe in detail the steps necessary to perform each analytical test. This may include but is not limited to: the sample, the reference standard and the reagents preparations, use of the apparatus, generation of the calibration curve, use of the formulae for the calculation, etc.

1.5.1.2. SPECIFICITY

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s).

This definition has the following implications: Identification: to ensure the identity of an analyte.

Purity Tests: to ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte, i.e. related substances test, heavy metals, residual solvents content, etc.

Assay (content or potency):

To provide an exact result which allows an accurate statementon the content or potency of the analyte in a sample.

1.5.1.3. ACCURACY

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.This is sometimes termed trueness.

1.5.1.4. PRECISION

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution.

The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

1.5.1.5. Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

1.5.1.5.1. Intermediate precision

Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

1.5.1.5.2. Reproducibility

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).

1.5.1.6. DETECTION LIMIT

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

1.5.1.7. QUANTITATION LIMIT

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

1.5.1.8. LINEARITY

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

1.5.1.9. RANGE

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

1.5.1.10. ROBUSTNESS

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

1.5.2 VALIDATION OF ANALYTICAL PROCEDURES: METHODOLOGY

INTRODUCTION

This document is complementary to the parent document which presents a discussion of the characteristics that should be considered during the validation of analytical procedures. Its purpose is to provide some guidance and recommendations on how to consider the various validation characteristics for each analytical procedure**.** In some cases (for example, demonstration of specificity), the overall capabilities of a number of analytical procedures in combination may be investigated in order to ensure the quality of the drug substance or drug product. In addition, the document provides an indication of the data which should be presented in a registration application.All relevant data collected during validation and formulae used for calculating validation characteristics should be submitted and discussed as appropriate.

Approaches other than those set forth in this guideline may be applicable and acceptable. It is the responsibility of the applicant to choose the validation procedure and protocol most suitable for their product. However it is important to remember that the main objective of validation of an analytical procedure is to demonstrate that the procedure is suitable for its intended purpose. Due to their complex nature, analytical procedures for biological and biotechnological products in some cases may be approached differently than in this document. Well-characterized reference materials, with documented purity, should be used throughout the validation study. The degree of purity necessary depends on the intended use.

In accordance with the parent document, and for the sake of clarity, this document considers the various validation characteristics in distinct sections. The arrangement of these sections reflects the process by which an analytical procedure may be developed and evaluated.

In practice, it is usually possible to design the experimental work such that the appropriate validation characteristics can be considered simultaneously to provide a sound, overall knowledge of the capabilities of the analytical procedure, for instance: specificity, linearity, range, accuracy and precision.

SPECIFICITY

An investigation of specificity should be conducted during the validation of identification tests, the determination of impurities and the assay. The procedures used to demonstrate specificity will depend on the intended objective of the analytical procedure.

It is not always possible to demonstrate that an analytical procedure is specific for a particular analyte (complete discrimination). In this case a combination of two or more analytical procedures is recommended to achieve the necessary level of discrimination.

Identification

Suitable identification tests should be able to discriminate between compounds of closely related structures which are likely to be present. The discrimination of a procedure may be confirmed by obtaining positive results (perhaps by comparison with a known reference material) from samples containing the analyte, coupled with negative results from samples which do not contain the analyte. In addition, the identification test may be applied to materials structurally similar to or closely related to the analyte to confirm that a positive response is not obtained. The choice of such potentially interfering materials should be based on sound scientific judgement with a consideration of the interferences that could occur.

Assay and Impurity Test (s)

For chromatographic procedures, representative chromatograms should be used to demonstrate specificity and individual components should be appropriately labelled. Similar considerations should be given to other separation techniques.

Critical separations in chromatography should be investigated at an appropriate level. For critical separations, specificity can be demonstrated by the resolution of the two components which elute closest to each other.

In cases where a non-specific assay is used, other supporting analytical procedures should be used to demonstrate overall specificity. For example, where a titration is adopted to assay the drug substance for release, the combination of the assay and a suitable test for impurities can be used.

The approach is similar for both assay and impurity tests

Impurities are available

For the assay, this should involve demonstration of the discrimination of the analyte in the presence of impurities and/or excipients; practically, this can be done by spiking pure substances (drug substance or drug product) with appropriate levels of impurities and/or excipients and demonstrating that the assay result is unaffected by the presence of these materials (by comparison with the assay result obtained on unspiked samples).

For the impurity test, the discrimination may be established by spiking drug substance or drug product with appropriate levels of impurities and demonstrating the separation of these impurities individually and/or from other components in the sample matrix.

Impurities are not available

If impurity or degradation product standards are unavailable, specificity may be demonstrated by comparing the test results of samples containing impurities or degradation products to a second well-characterized procedure e.g. pharmacopoeial method or other validated analytical procedure (independent procedure). As appropriate, this should include samples stored under relevant stress conditions: light, heat, humidity, acid/base hydrolysis and oxidation.

- for the assay, the two results should be compared;
- for the impurity tests, the impurity profiles should be compared.

Peak purity tests may be useful to show that the analyte chromatographic peak is not attributable to more than one component (e.g., diode array, mass spectrometry).

LINEARITY

A linear relationship should be evaluated across the range (see section 3) of the analytical procedure. It may be demonstrated directly on the drug substance (by dilution of a standard stock solution) and/or separate weighings of synthetic mixtures of the drug product components, using the proposed procedure. The latter aspect can be studied during investigation of the range.

Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares. In some cases, to obtain linearity between assays and sample concentrations, the test data may need to be subjected to a mathematical transformation prior to the regression analysis. Data from the regression line itself may be helpful to provide mathematical estimates of the degree of linearity.

The correlation coefficient, y-intercept, slope of the regression line and residual sum of squares should be submitted. A plot of the data should be included. In addition, an analysis of the deviation of the actual data points from the regression line may also be helpful for evaluating linearity.Some analytical procedures, such as immunoassays, do not demonstrate linearity after any transformation. In this case, the analytical response should be described by an appropriate function of the concentration (amount) of an analyte in a sample. For the establishment of linearity, a minimum of 5 concentrations is recommended. Other approaches should be justified.

RANGE

The specified range is normally derived from linearity studies and depends on the intended application of the procedure. It is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure.

The following minimum specified ranges should be considered:

- for the assay of a drug substance or a finished (drug) product: normally from 80 to 120 percent of the test concentration;
- for content uniformity, covering a minimum of 70 to 130 percent of the test concentration, unless a wider more appropriate range, based on the nature of the dosage form (e.g., metered dose inhalers), is justified;
- for dissolution testing: +/-20 % over the specified range;

e.g., if the specifications for a controlled released product cover a region from 20%, after 1 hour, up to 90%, after 24 hours, the validated range would be 0-110% of the label claim.

- for the determination of an impurity: from the reporting level of an impurity to 120% of the specification; for impurities known to be unusually potent or to produce toxic or unexpected pharmacological effects, the detection/quantitation limit should be commensurate with the level at which the impurities must be controlled;

Note: for validation of impurity test procedures carried out during development, it may be necessary to consider the range around a suggested (probable) limit.

- if assay and purity are performed together as one test and only a 100% standard is used, linearity should cover the range from the reporting level of the impurities to 120% of the assay specification.

ACCURACY

Accuracy should be established across the specified range of the analytical procedure.

Assay

Drug Substance

Several methods of determining accuracy are available

a) application of an analytical procedure to an analyte of known purity (e.g. reference material)

b) comparison of the results of the proposed analytical procedure with those of a second well-characterized procedure, the accuracy of which is stated and/or defined (independent procedure, see 1.2.)

c) accuracy may be inferred once precision, linearity and specificity have been established.

Drug Product

Several methods for determining accuracy are available

a) application of the analytical procedure to synthetic mixtures of the drug product components to which known quantities of the drug substance to be analysed have been added

b) in cases where it is impossible to obtain samples of all drug product components , it may be acceptable either to add known quantities of the analyte to the drug product or to compare the results obtained from a second, well characterized procedure, the accuracy of which is stated and/or defined (independent procedure, see 1.2.)

c) accuracy may be inferred once precision, linearity and specificity have been established.

Impurities (Quantitation)

Accuracy should be assessed on samples (drug substance/drug product) spiked with known amounts of impurities.

In cases where it is impossible to obtain samples of certain impurities and/or degradation products, it is considered acceptable to compare results obtained by an independent procedure (see 1.2.). The response factor of the drug substance can be used.It should be clear how the individual or total impurities are to be determined e.g., weight/weight or area percent, in all cases with respect to the major analyte.

Recommended Data

Accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g., 3 concentrations or 3 replicates each of the total analytical procedure).

Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals.

PRECISION

Validation of tests for assay and for quantitative determination of impurities includes an investigation of precision.

Repeatability

Repeatability should be assessed using

a) a minimum of 9 determinations covering the specified range for the procedure (e.g., 3 concentrations/3 replicates each)

b) a minimum of 6 determinations at 100% of the test concentration.

Intermediate Precision

The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. The applicant should establish the effects of random events on the precision of the analytical procedure. Typical variations to be studied include days, analysts, equipment, etc. It is not considered necessary to study these effects individually. The use of an experimental design (matrix) is encouraged.

Reproducibility

Reproducibility is assessed by means of an inter-laboratory trial. Reproducibility should be considered in case of the standardization of an analytical procedure, for instance, for inclusion of procedures in pharmacopoeias. These data are not part of the marketing authorization dossier.

Recommended Data

The standard deviation, relative standard deviation (coefficient of variation) and confidence interval should be reported for each type of precision investigated.

DETECTION LIMIT

Several approaches for determining the detection limit are possible, depending on whether the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

Based on Visual Evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods. The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

Based on Signal-to-Noise

This approach can only be applied to analytical procedures which exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

Based on the Standard Deviation of the Response and the Slope

The detection limit (DL) may be expressed as

$$DL = \frac{3.3\,\sigma}{S}$$

where σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimate of σ may be carried out in a variety of ways, for example

Based on the Standard Deviation of the Blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

Based on the Calibration Curve

A specific calibration curve should be studied using samples containing an analyte in the range of DL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

Recommended Data

The detection limit and the method used for determining the detection limit should be presented. If DL is determined based on visual evaluation or based on signal to noise ratio, the presentation of the relevant chromatograms is considered acceptable for justification.

In cases where an estimated value for the detection limit is obtained by calculation or extrapolation, this estimate may subsequently be validated by the independent analysis of a suitable number of samples known to be near or prepared at the detection limit.

QUANTITATION LIMIT

Several approaches for determining the quantitation limit are possible, depending on whether the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

Based on Visual Evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods.

The quantitation limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

Based on Signal-to-Noise

This approach can only be applied to analytical procedures that exhibit baseline noise.

Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1.

Based on the Standard Deviation of the Response and the Slope

The quantitation limit (QL) may be expressed as:

$$QL = \frac{10\,\sigma}{S}$$

where σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimate of σ may be carried out in a variety of ways for example:

Based on Standard Deviation of the Blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

Based on the Calibration Curve

A specific calibration curve should be studied using samples, containing an analyte in the range of QL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

Recommended Data

The quantitation limit and the method used for determining the quantitation limit should be presented. The limit should be subsequently validated by the analysis of a suitable number of samples known to be near or prepared at the quantitation limit.

ROBUSTNESS

The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters.If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure. One consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g., resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used.

Examples of typical variations are

- stability of analytical solutions;
- extraction time.

In the case of liquid chromatography, examples of typical variations are

- influence of variations of pH in a mobile phase,
- influence of variations in mobile phase composition,
- different columns (different lots and/or suppliers),
- temperature,
- flow rate.

In the case of gas-chromatography, examples of typical variations are

- different columns (different lots and/or suppliers)
- temperature

- flow rate.

1.6.1. SYSTEM SUITABILITY TESTING

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated. See Pharmacopoeias for additional information.

Acceptance criteria of validation for HPLC

S.NO.	Characteristics	Acceptance Criteria
1	Accuracy	Recovery 98 - 102% with 80,100 and 120% of spiked samples
2	Precision a)Repeatability b) Intermediate precision	 RSD < 2% RSD < 2%
3	Specificity/selectivity	No interference
4	Detection limit	S/N > 2 or 3
5	Quantization limit	S/N > 10
6	Linearity	r > 0.999
7	Range	80 - 120%
8	Stability	> 24h or > 12h

1.6.2 SYSTEM SUITABILITY PARAMETERS

(USP XXIII 1995; Beckett and Stenlake, 2002; Willard, 1986)

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment , electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such system

suitability test parameters to be established for a particular procedure depend on the type of procedures being validated.

The parameters that are affected by the changes in chromatographic conditions are

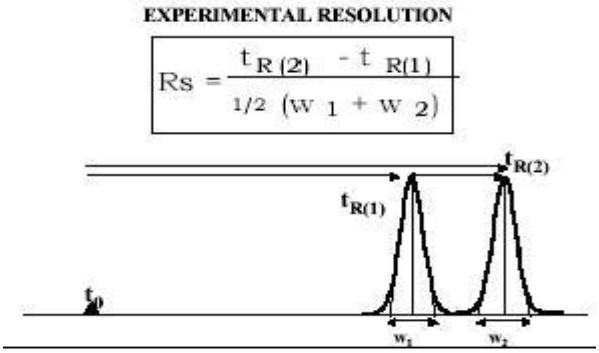
1) Retention time (t_R)
2) Resolution (R_S)
3) Capacity factor ($k`$)
4) Selectivity (α)
5) Number of Theoretical plates (N)
6) HETP
7) Asymmetry factor
8) Tailing factor

1) Retention time

Chromatographic retention is to measure the time between the injection point and maximum of the detector response for correspondent compound. This parameter called "retention time" is inversely proportional to the eluent flow rate.

2) Resolution

The degree of separation or resolution of two adjacent band is defined as the distance between band peaks divided by the average band width retention and band width are measured in units of time.



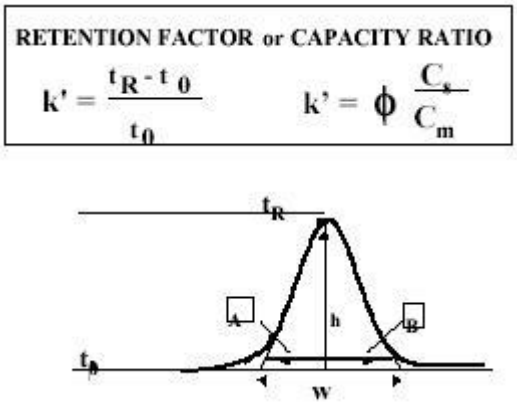
Where,

Rt_1 and Rt_2 are the retention times of components 1 and 2

W_1 and W_2 are peak widths of components 1 and 2.

3) Capacity factor (K')

It is a measure of a sample peak in the chromatogram being specific for a given compound, a parameter which specifies of a substance to be separated.

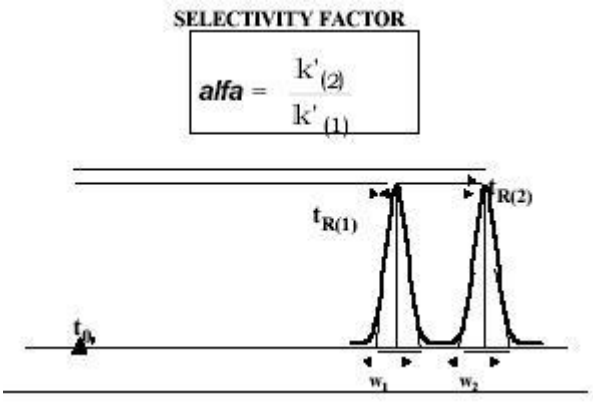


4) Selectivity (α)

The selectivity α is a measure of relative retention of two components in a mixture. The ideal value of selectivity is 2. It can be calculated by using the following formula.

$$\alpha = \frac{V_2 - V_0}{V_1 - V_0}$$

Where, V_0 is the void volume of the column and V_2 and V_1 are the retention volumes of the second and the first peak, respectively.



5) Number of Theoretical plates (N)

The number of theoretical plates N is a measure of column efficiency. For Gaussian peaks it is calculated by the following formula.

$$N = 16 \frac{Rt^2}{W^2}$$

Where t = retention time

W = width of the peak its base obtained by the extrapolating relatively straight sides of the peak to the base line.

$W_{1/2}$ = width of the peak at the half height obtained directly by electronic integrators.

The value of N depends upon the substance being chromatographed as well as the operating conditions such as mobile phase, temperature.

Limit = N > 2000 is desirable.

6) Height equivalent to a Theoretical plate (HETP)

A theoretical plate can be of any height which decides the efficiency of separation. If HETP is less the column is more efficient. If HETP is more the column efficiency is less the equivalent to a theoretical plate (HETP) is given by

$$HETP = \frac{Length\ of\ column}{n}$$

Where n = Number of theoretical plates.

7) Peak asymmetry

Peak asymmetry factor as can be used as a criterion of column performance. The peak half width, divided by the corresponding front half width, a gives the asymmetry factor

8) Tailing factor:

The tailing factor T, a measure of peak symmetry, is unity for perfectly symmetrical peaks and its value increases as tailing becomes more pronounced.

In some cases, values less than unity may be observed. As peak asymmetry increases, integration, hence precision becomes less reliable.

$$T = \frac{W_{0.05}}{2f}$$

Where, W0.05 = Width of peak at 5% height.

F = distance from the peak maximum to the leading edge of the peak. The distance being measured at a point 5% of the peak from the base line.

Limit ≤ 2 is preferable.

1.7 STATICAL PARAMETERS *(*Mendham et al. 2002)

1.7.1. Linear regression

Once linear relationship has been shown to have a high probability by the value of the correlation coefficient 'r' then the best straight line through the data points has to be estimated. This can be often done by visual inspection of graph but in many cases it is far more sensible to evaluate the best straight line by linear regression.

The equation of straight line is

$$Y = mx + c$$

Where, y the dependent variable is plotted as result changing x the independent variable.

To obtain regression line ‘y ‘ on ‘x ‘ the slope ‘m’ of the line intercept ‘c’ on the y axis are given by the following formula.

$$m = \frac{N\sum xy - (\sum x)(\sum y)}{N\sum x^2 - (\sum x)^2}$$

$$c = \frac{(\sum y)(\sum x^2) - (\sum x)(\sum xy)}{N\sum x^2 - (\sum x)^2}$$

1.7.2. Correlation coefficient

The correlation co-efficient is used as a measure of the correlation between two variables. When variables x and y are correlated rather than being functionally related. The person correlation co efficient is one of the most convenient to calculate. This is given by

$$r = \frac{n\sum x_1 y_1 - \sum x_1 y_1}{\{[n\sum x_1{}^2 - (\sum x_1{}^2][n\sum y_1{}^2 - (\sum y_1{}^2]\}^{1/2}}$$

Where n is the number of data points.

The maximum, value of r is 1. When this occurs, there is exact correlation between the two variables. When r is zero, there is complete independence of the variables. The minimum value of r is -1. A negative correlation co-efficient indicates that the assumed dependence is opposite to what exists and therefore a positive co-efficient for the revered relation. The fit must be quite poor before r become smaller than about 0.98 and is really very poor when less than 0.9.

1.7.3. Standard deviation

It is commonly used in statistics as a measure of precision and is more meaning full than is the average deviation. It may be thought of as a root mean square deviation of values from their average and is expressed mathematically as

Where, S = Standard deviation $S = \sqrt{\frac{\sum_{i=1}^{i=n}(x - \bar{x})}{N-1}}$

If N is large (50 or more) then of course it of immaterial whether the term in the denomination is N - 1 or N.

Σ = sum

$\bar{x}$ = Mean or arithmetic average.

$x - \bar{x}$ = Deviation of a value from the mean

N = Number of observations

1.7.4. Percentage relative standard deviation (% RSD)

It is also known as co efficient of variation CV. It is defined as the standard deviation (S.D) expressed as the percentage of mean.

$$C V \text{ or } \% \text{ RSD} = \frac{S.D}{\bar{x}} \times 100$$

Where

S.D = Standard deviation

The variance is defined as S^2 and is more important in statistics than S itself. However the latter is much more commonly used with chemical data.

1.7.5. Standard error of mean (S.E)

The standard error of mean can be defined as the value obtained by the division of standard deviation by square root of number of observations. It is mathematically expressed as,

$$S.E. = \frac{S.D}{\sqrt{n}}$$

Where,

S.D = Standard deviation.

n = number of observations.

LITERATURE REVIEW

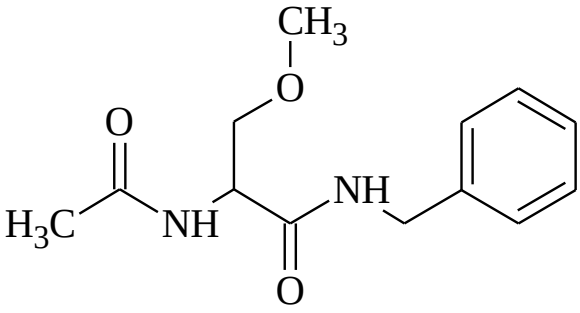
2. REVIEW OF LITERATURE

2.1. DRUG PROFILE

(*The Merck index 2006, http://toxwiki.wikispace/lacosamide.com, www.drugs.com/ppa/lacosamide.html)*

LACOSAMIDE

Molecular Structure



Chemical Structure

(2R)-2-(acetylamino)-N-benzyl-3-methoxypropanamide

Molecular Formula

$C_{13}H_{18}N_2O_3$

Molecular Weight

250.30 g/ mol

Description

White to slightly yellow crystalline powder

Category

Anti convulsant

Storage

Tablet and liquid should be stored at 20-25 ^{0}C

Solubility

Freely soluble in water and in organic solvents such as Ethanol, Methanol, dimethyl formamide, dimethyl sulphoxide, slightly soluble in acetonitrile and soluble in phosphate buffered saline at pH 7.2. Practically insoluble in 0.1 M Sodium Hydroxide

Identification

1. Melting point

Drug	Standard value	Observed value*
Lacosamide	143-144˚C	143 ˚C

*****Average of six determination.

2. IR spectra was recorded and shown in figure 1.

Indication

Lacosamide is indicated as a adjunctive therapy in the treatment of partial-onset seizure with or without secondary generalization in patient with epilepsy aged sixty years and olders, it is also being developed for the treatment of diabetic neuropathy pain.

Pharmacodynamics

Lacosamide has a novel dual mechanism of action. it selectively enhances slow inactivation of voltage-gated sodium channels. Stabilizing hyperexcitable neuronal membranes and inhibiting neuronal firing. In addition . it modulates collapsing response mediator protein-2(CRMP-2). The role of CRMP-2 in seizures has not been fully elucidated, however , its expression is altered in epilepsy and other neurodegenerative disease.

Pharmacokinetics

Absorption

Lacosamide is rapidly completely absorbed after oral administration. The oral bioavailability of Lacosamide tablet is approximately 100%. Following oral administration, the plasma concentration of unchanged Lacosamide increases rapidly and reach.es C_{max} about 0.5-4 hours post dose. The food does not affect the rate and extended of absorption. The plasma concentration increases proportional with dose after oral and i.v administration.

Distribution

The volume of distribution is approximately 0.6 L/kg. Lacosamide is less than 15% bound to plasma protein

Metabolism

Lacosamide undergoes demethylation to o- desmethyl Lacosamide, an inactive metabolite. 95% of dose is excreted in the urine as drug and metabolites.the major compounds excreted in urine are unchanged Lacosamide (approximately 40% of the dose) and its o-desmethyl Lacosamide metabolites less than 30%

Elimination

Lacosamide is primarily eliminated from the systemic circulation by renal excretion and biotransformation. After oral and intravenous administration of Lacosamide. The elimination half life is unchanged drug is approximately 13 hours. Approximately 95% Lacosamide administered was recovered in the urine and less than 0.5% in the faeces.

Dosage and Administration

The recommended initial dose is 50 mg / twice a day upto a maintenance dose of 200-400 mg/day.

Side Effects

Blurred vision; dizziness; drowsiness; headache; irritability; mild itching; pain, or redness at the injection site; nausea; tiredness; tremor; vomiting; weakness.

Drug Interactions

The interaction with several drug has been studied. No interaction between Lacosamide and carbamazepine valproic acid, omeprazole, metforminl, digoxin and oral contraceptive containing ethinyl estrodiol and levonorgestrel has been observed. Additionally clinicall studies have have shown that Lacosamide does not affect plasma level of concomitantly administered carbamazepine levetiracetam lamotrigin, topiramate, valproate, and phenytoin. In in vitro studies revealed that Lacosamide at concentration 15 time greater than therapeutic plasma level, inhibited cyp 2c19, however plasma concentration were similar in poor and extensitive cyp 2c19 metabolizer and in individual with inhibited cyp 2c19.

2.2. REPORTED METHOD

2.2.1 Kestlyn .C *et al.,* (2011) Reported "A Simple HPLC-UV Method for the Determination of Lacosamide in Human Plasma". This chromatographic method was achieved on ACE C18-AR column (2.1 mm x 150mm, 3.0 µm)and mobile phases consisting of mixtures of ammonium formate buffer at pH 9 and acetonitrile .

2.2.2 Usmangani .K Chhalotiya *et al.,* (2011) Reported **"**Scientific Paper Stability-Indicating Liquid Chromatographic Method for Quantification of new Anti Epileptic Drug Lacosamide in Bulk and Pharmaceutical Formulations". This chromatography was achieved on hypersil C-18, 4.5 mm column, mobile phase containing Acetonitrile: Water (20:80 v/v) was used.

2.2.3 Clare Greenaway *et al*., (2011) Reported "Lacosamide saliva and serum concentrations in patients with epilepsy" These data support the use of saliva as a viable alternative to serum for monitoring Lacosamide therapy in patients with epilepsy. Lacosamide concentrations in serum (free and total) and in saliva were determined by high performance liquid chromatography (HPLC).Results:Linear regression analysis showed a good correlation between Lacosamide dose and both total (r^2 = 0.825; n = 32) and free (r^2 = 0.815; n = 29) serum concentrations, and Lacosamide serum total and free concentrations were linearly related (r^2 = 0.721; n = 97). There was also a good correlation between saliva Lacosamide and both total (r^2 = 0.842; n = 49) and free (r^2 = 0.828; n = 47) serum Lacosamide concentrations.

2.2.4 Soo-Jin Kim *et al.* (2011) reported "Liquid Chromatography-Tandem Mass Spectrometry for Quantification of Lacosamide, an Antiepileptic Drug, in Rat Plasma and its Application to Pharmacokinetic Study". The method involves the addition of acetonitrile and internal standard solution to plasma samples,

followed by centrifugation. An aliquot of the supernatant was diluted with water and directly injected into the LC/MS/MS system. The separations were performed on column packed with octadecylsilica (5 µm, 2.0 × 50 mm) with 0.1% formic acid and acetonitrile as mobile phase, and the detection was performed on tandem mass spectrometry by the multiple-reaction monitoring via an electrospray ionization source.

2.2.5 Martinez *et al.,* (2011) Reported "High-Performance Liquid Chromatographic Analysis of Lacosamide in Canine Serum using Ultraviolet Detection: Application to Pre-Clinical Pharmacokinetics in Dogs". A novel and simple high-performance liquid chromatography method was developed for determination of lacosamide in dog serum. Serum proteins (0.1 mL) were precipitated with -20.0°C acetonitrile after addition of the internal standard, daidzein. Separation was achieved with a Phenomenex® Luna® C(18) (2) (5 µm, 250 × 4.60 mm) column with ultraviolet detection at 210 nm.

2.2.6 Kalyan chakravathy *et al.,* (2011) reported "Development and Validation of RP-HPLC Method for Estimation of Lacosamide in Bulk and its Pharmaceutical Formulation". This chromatography was achieved with a Develosil ODS HG-5 Column and sodium dihydrogen phosphate monohydrate buffer :Acetonitrile (700: 300 v/v) as mobile phase flow rate 1.0 mL/ min Detection was performed at 210 nm

2.2.7 Sai sumanth *et al.,* (2012) reported "Validated Spectrophotometric Estimation of Lacosamide in Bulk and Tablet Dosage Forms" using Water as solvent. Lacosamide has the absorbance maxima at 257 nm. Beer's Law was found to be obeyed in the concentration range 300- 900 µg/ mL Result of the Analysis were validated statistically and by Recovery studies.

Aim and plan of work

3. AIM AND PLAN OF WORK

3.1 Aim of Work

The drug analysis plays an important role in the development, manufacture and therapeutic use of drug. Most of the pharmaceutical industries do the quantitative chemical analysis to ensure that the raw material used and the final product thus obtained meet certain specifications and to determine how much of each components are present in the final product.

Standard analytical procedure for newer drugs or formulation may not be available in pharmacopoeias hence it is essential to develop newer analytical methods which are accurate, precise, specific, linear, simple and rapid. UV and HPLC grades of solvents used for respective determination and solvent should readily available and cheaper. The solvent should be completely extracting the active ingredient from formulation.

Lacosamide is indicated as a adjunctive therapy in the treatment of partial-onset seizure with or without secondary generalization in patient with epilepsy aged sixty years and older, it is also being developed for the treatment of diabetic neuropathy pain. This is a newer drug in Indian market and CDSCO approves this newer drug on December 2010.

Extensive literature survey revealed that the HPLC – UV method for the determination of Lacosamide in plasma has been reported. However there is no evidence for the estimation of Lacosamide by UV spectrophotometry, HPTLC and HPLC - UV in bulk and in tablet formulation.

Hence the present work, aims to develop a simple, precise and accurate methods for the estimation of Lacosamide in bulk and in pharmaceutical dosage form and to validate the developed methods by using UV spectroscopy, HPTLC and

RP – HPLC method. The developed HPLC method was compared with the reported methods.

3.2 Plan of Work

3.2.1 Survey on literature

The survey on literature performed for Lacosamide for their physiochemical properties, solubility, pharmacology and analytical techniques. So this basic information gives notation for newer method development.

3.2.2 Method development

1) Identification of drug by its melting point.

2) Determination of solubility of the drugs.

3) Development of simple, cost effective and accurate spectrophotometric methods.

4) Development of simple, cost effective and accurate HPTLC method.

5) Development of a rapid and accurate RP – HPLC method involving UV - detection.

6) Analysis of marketed formulations.

7) Validation of developed analytical methods.

8) Statistical analysis of developed analytical methods.

3.2.3 Validation of developed method

The developed method should be validated as per ICH guidelines. The parameters used to validate the developed method are Accuracy, Precision, Specificity, Limit of Detection, Limit of Quantification, Linearity, Range, Robustness and Ruggedness. The system suitability parameters like Capacity factor, Asymmetric factor, Tailing factor, Number of theoretical plates, HETP and Resolution should be calculated for RP-HPLC chromatograms and compare with standard values.

MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1 Materials

4.1.1 Drug Samples (Raw material)

Lacosamide was obtained as a gift sample from Alkem pharma, Mumbai.

4.1.2 Formulation used

LACOSAM tablets (Torrent pharmaceuticals, Mumbai) containing Lacosamide 100 mg was procured from OM pharmacy, Chennai.

4.1.3 Chemicals and solvents used

Distilled water, Potassium dihydrogen orthophosphate (AR Grade), Methanol (HPLC grade), Water (HPLC grade), Acetonitrile (HPLC grade), were purchased from Qualigens India Pvt. Limited and Loba Chemie India Limited, Mumbai.

4.1.4 Instruments used

Different instruments used to carry out the present work,

1) Shimadzu AUX - 220 Digital balance
2) Shimadzu - 1700 Double Beam UV - Visible spectrophotometer with pair of 10 mm matched quartz cells
3) ELICO SL - 210 Double Beam UV - Visible spectrophotometer with pair of 10 mm matched quartz cells
4) ELICO pH meter (Model LI - 120)
5) CAMAG - HPTLC Instrument
6) THERMO SCIENTIFIC SPECTRA - HPLC system
7) SOLTECH – Sonica ultrasonic cleaner – Model 2200 MH
8) REMI – Centrifuge apparatus
9) CYBERLAB – Micropipette

Specifications (Terms) of instruments

a) Shimadzu AUX - 220 Digital balance (Shimadzu Instruction Manual)

Specifications	
Weighing capacity	200 gms
Minimum display	0.1 mg
Standard deviation	≤ 0.1 mg
Operation temperature range	5 to 40° C

b) Double Beam UV - Visible Spectrophotometer (Shimadzu and ELICO Instruction Manuals)

Model: Shimadzu UV - 1700; Double beam UV - Visible spectrophotometer.

ELICO SL – 210; Double beam UV - Visible spectrophotometer.

Specification	**Shimadzu UV - 1700**	**Elico SL - 210**
Light source	20 W halogen lamp, Deuterium lamp, Light source position automatic adjustment mechanism. Built in lamp lighting time display function.	Tungsten halogen lamp (W), Deuterium lamp (D), Light source position automatic adjustment mechanism.

Monochromator	Aberration- correcting concave blazed holographic grating	Concave holographic grating with 1200 lines/ mm
Detector	Silicon photodiode	Photodiode
Stray Light	0.04% or less (220 nm; NAI 10g/ lt) 0.04% or less (340 nm; $NaNo_2$ 50g/ lt).	< 0.05% T at 220 nm with NAI 10g/ lt
Measurement Wavelength range	190 ~ 1100 nm	190 ~1100 nm
Spectral Band width	1 nm or less (190 to 900nm).	1.8 nm
Wave length Accuracy	± 0.5 nm on broad automatic wavelength calibration mechanism.	± 0.5 nm automatic wavelength calibration mechanism.
Recording range	Absorbance; - 3.99 ~ 3.99 Abs Transmittance; - 399 ~ 399%	Absorbance; ± 3.000 Abs
Photometric accuracy	± 0.004 Abs (at 1.0 Abs). ± 0.002 Abs (at 0.5 Abs).	± 0.005 Abs (at 1.0 Abs). ± 0.010 Abs (at 0.5 Abs).
Operating Temperature/ Humidity	Temperature range; 15 to 35˚C Humidity range; 35 to 80% (15 to below 35˚C) 35 to 70% (30 to below 35˚C)	Temperature range; 15 to 35˚C Humidity range; 35 to 80% (15 to below 35˚C) 35 to 70% (30 to below 35˚C)

INSTRUMENT SPECIFICATION FOR HPLC

THERMO SCIENTIFIC SPECTRA HPLC system	
Pump	p4000
auto sampler	as3000
UV-VIS detector	uv 2000
vaccum degasser	scm 1000
system controller	sn 4000
sample cooler	up to 2-4° c
soft ware	chrom quest 5.0

A) HPTLC INSTRUMENT – CAMAG SPECIFICATION

Sample Applicator	Linomat IV with Camag 100 µL Syringe
Chamber	Camag Twin trough glass chmber (20×
Scanner	Camag TLC Scanner III
Software	Wincat software
Development temperature	Ambient
Stationary Phase	HPTLC plates (Merck) precoated with silica gel 60 F 254 on Aluminium sheets
Development time	20 min

4.2 METHODS

In the present work an attempt was made to develop and validate simple, precise and accurate methods for the estimation of Lacosamide in pure form and in tablet dosage form by

1) First Order Derivative Spectrophotometric Method
2) RP – HPLC Method
3) HPTLC method

4.2.1 First Order Derivative Spectroscopic Method

This method involves the conversion of normal spectra to its first derivative spectrum. The absorption spectra is referred to as the Fundamental, zeros order or D^o spectrum. The first derivative (D^o) spectrum is a plot of the state of change of absorbance with wavelength, i.e a plot of the slope of the fundamental spectrum against wavelength or a plot of $da/ d\lambda$ vs λ The absorbances of a number of standard solutions of the reference substance of the concentration encomparing the sample concentration are measured and a calibration graph is constructed. The concentration of the sample solution is read from the graph as the concentration corresponding to the absorbance of the solution

4.1.2.1. Selection of solvent

The solubility of Lacosamide was determined in a variety of solvents as per Indian pharmacopoeia standards. Solubility was carried out in polar and non polar solvents. From the solubility data Distilled Water was selected as solvent for the analysis of Lacosamide.

4.1.2.2.Preparation of standard stock solution

10 mg of Lacosamide raw material was weighed accurately and transferred in to 10 mL volumetric flask and dissolved in Distilled Water and made up to the volume with Distilled Water. This solution contains 1000 µg / mL concentration.

4.1.2.3.Selection of wavelengths for estimation and stability studies

The standard stock solution was further diluted with Distilled Water to get the concentration of 10 µg/ mL and the solution was scanned between 200 and 400 nm

using Distilled Water as blank. The spectra was derivatised to first order and from the spectra, 216.5 nm was selected as an analytical wavelength.

The stability was performed by measuring the solution at different time intervals. It was observed that Lacosamide in Distilled Water was stable for up to 4 hours at the selected wavelength.

4.1.2.4.Preparation of calibration graph

The standard stock solution of Lacosamide (1mL – 5 mL) was transferred into series of 100 mL volumetric flasks and made up to the volume with Distilled Water. The absorbance of different concentration solutions were measured at 216.5 nm. The calibration curve was constructed by plotting concentration Vs absorbance. Lacosamide was linear with the concentration range of 10 – 50 μg/ mL at 216.5 nm.

4.1.2.5.Quantification of raw material

3 mL of standard stock solution was taken in to series of six 100 mL standard flasks and the volume was made up to mark with Distilled Water. The absorbance of these solutions was measured at 216.5 nm. The amount Lacosamide present in the raw material was determined by using slope and intercept values from calibration graph.

4.1.2.6.Quantification of formulation

10 tablets of formulation (LACOSAM) containing Lacosamide equivalent to 100 mg were weighed accurately and the average weight of each tablet was found. The tablets were ground to a fine powder. The tablet powder equivalent to 50 mg of Lacosamide was weighed and transferred into 50 mL volumetric flask. Added a minimum quantity of Distilled Water to dissolve the substance and the solution was sonicated for 15 minutes. The volume was made up to 50 ml with distilled water. The solution was centrifuged for 15 minutes and the supernatant liquid was filtered through Whatmann filter paper No. 41. From the clear solution, further dilutions were

made by diluting 3 mL into 100 mL with Distilled Water to obtain 30 μg/ mL solution theoretically. The absorbances of six replicates were measured and the amount was calculated by using regression equation. This procedure was repeated for six times.

4.1.2.7. Recovery studies

a) Preparation of Lacosamide raw material stock solution

100 mg of Lacosamide was accurately weighed and transferred into 10 mL Volumetric flask and sufficient Distilled Water was added to dissolve the substance and made up to the mark with the same. This contains 10 mg / mL concentration.

b) Procedure

The recovery experiment was done by adding known concentrations of raw material stock solution of Lacosamide to the preanalyzed formulation. The tablet powder equivalent to 50 mg of Lacosamide was weighed accurately and added 4mL, 5 mL and 6 mL of above raw material stock solution into a series of 50 mL standard flasks and dissolved with Distilled Water and the solution was sonicated for 15 minutes. The volume was made up to 50 mL with distilled water. The solution was centrifuged for 15 minutes at 2000 rpm and the supernatant liquid was filtered through a Whatmann filter paper No.41. 3 mL of the clear solution was transferred into 100 mL volumetric flask and made up to 100 mL with Distilled Water. The absorbance of three replicates was measured at the selected wavelength. The amount of drug recovered from formulation was calculated. The procedure was repeated for three times for each concentration.

4.1.2.7.1. Validation of developed method

4.1.2.7.2. Linearity

A calibration curve was plotted between concentration and absorbance. Lacosamide was linear in the concentration range of 10 – 50 µg/ mL of Lacosamide at 216.5 nm.

4.1.2.7.3.Precision

The repeatability of the method was confirmed by the analysis of formulation was repeated for six times with the same concentration. The amount of drug present in the tablet formulation was calculated. The percentage RSD was calculated. The intermediate precision of the method was confirmed by intraday and interday analysis i.e. the analysis of formulation was repeated three times in the same day and on three successive days. The amount of drugs was determined and the percentage RSD also calculated.

4.1.2.7.3. Accuracy (Recovery studies)

Accuracy of the method was confirmed by recovery studies. To the preanalyzed formulation, a known quantity of raw material of Lacosamide was added and the procedure was followed as per the analysis of formulation. The amount of each drug recovered was calculated. This procedure was repeated for three times for each concentration. The % RSD was calculated.

4.1.2.7.4. Ruggedness

Ruggedness of the method was confirmed by the analysis of formulation was done by using different instruments and different analysts. The amount and % RSD were calculated.

4.1.2.7.5. LOD and LOQ

The linearity study was carried out for six times. The LOD and LOQ were calculated based upon the calibration curve method.

The LOD and LOQ were calculated by using the average of slope and standard deviation of intercept.

4.2.3 REVERSE PHASE – HPLC METHOD

4.2.3.1. Selection of chromatographic method

Proper selection of the method depends upon the nature of sample, polarity, molecular weight, pka value and solubility. The drug Lacosamide for the present study was polar. So, reverse phase chromatographic technique was selected by using Intersil ODS- 3V column as a stationary phase with different ratios of 50Mm Potassium dihydrogen orthophosphate: Acetonitrile: Methanol as a mobile phase.

4.2.3.2. Preparation of mobile phase

The mobile phase was prepared by mixing Acetonitrile with 50 mM Potassium Dihydrogen Ortho Phosphate and Methanol in the ratio of 10: 30: 60 % v/v and sonicated for 15 minutes to degas the mobile phase.

4.2.3.3. Method development and optimization of chromatographic conditions

4.2.3.3.1. Preparation of standard stock solution for RP-HPLC

100 mg of Lacosamide raw material was accurately weighed and transferred into 100 mL volumetric flask and dissolved in methanol (HPLC grade), after dissolution the volume was made up to the mark with methanol (HPLC grade). The solution was observed to contain 1000 µg/ mL.

4.2.3.3.2. Selection of Detection wavelength

Solution of Lacosamide (10 µg/ mL) was made by diluting the stock solution in the mobile phase of 50mM Potassium Dihydrogen Orthophosphate: Acetonitrile:

Methanol (30: 10: 60 % v/v/v) and scanned in the UV region of 200 – 400 nm and recorded the spectrum. From spectra at 210 nm the drug showed maximum absorbance. Hence this was selected as a detection wavelength for better sensitivity.

4.2.3.3.3. Stability of sample solutions

The stability was performed by measuring the absorbance of same solution at different time intervals. It was found that Lacosamide was stable up to three hours.

4.2.3.4 Optimization of chromatographic conditions

4.2.3.4.1 Initial separation conditions

The following chromatographic conditions were preset initially to get better resolution of Lacosamide.

Mode of operation	-	Isocratic
Stationary phase	-	Intersil ODS- 3V Column (150 mm × 4.6 mm i.d. 5μ)
Mobile phase	-	50mM Potassium Dihydrogen Orthophosphate: Acetonitrile: Methanol.
Proportion of mobile phase	-	(50: 40: 10 % v/v/v)
Detection wavelength	-	210 nm
Flow rate	-	1 mL/ min
Temperature	-	Ambient
Sample load	-	20 μl
Operating pressure	-	2000 psi
Method	-	External Standard Calibration method

The mobile phase was primarily allowed to run for 60 minutes to record a steady baseline. The solution of Lacosamide was injected and the respective chromatogram was recorded. It was found that Lacosamide was eluted at 1.36 minutes

and the peak splitting was observed. For this reason different ratios of mobile phase with different solvents were tried to obtain good chromatogram with acceptable system suitability parameters.

4.2.3.4.2 Selection of mobile phase

Different mixtures of mobile phase with different ratios were selected and their chromatograms were recorded, they include the following

S.No	Mobile phase	Observation
1.	50mM Potassium Dihydrogen Orthophosphate: Acetonitrile: Methanol (50:40:10%v/v/v)	Lacosamide was eluted with less retention time. Peak splitting was observed.
2	50mM Potassium Dihydrogen Orthophosphate: Acetonitrile: Methanol (50: 20: 30% v/v/v)	A broad peak was observed.
3	50mM Potassium Dihydrogen Orthophosphate: Acetonitrile: Methanol (30:10:50%v/v/v)	Lacosamide was eluted with less retention time.

From the above information, in the mobile phase of 50mM Potassium dihydrogen orthophosphate: Acetonitrile: Methanol (30: 10: 60 % v/v/v), the drug was eluted with sharp peak. Hence this mobile phase was used for the analysis of Lacosamide.

4.2.3.4.3 Optimized Chromatographic Conditions

Mode of operation - Isocratic

Stationary phase - Intersil ODS- 3V Column

(150 mm × 4.6 mm i.d. 5μ)

Mobile phase	-	50mM Potassium Dihydrogen Orthophosphate: Acetonitrile: Methanol
Proportion of mobile phase	-	30: 10: 60 % v/v/v
Detection wavelength	-	210 nm
Flow rate	-	1 mL/ min
Temperature	-	Ambient
Sample load	-	20 µl
Operating pressure	-	2000 psi
Method	-	External Standard Calibration method

4.2.3.4.4 Preparation of standard stock solution

100 mg of Lacosamide raw material was weighed and transferred into 100 mL volumetric flask, dissolved in methanol (HPLC grade) and made up to the volume with same. The solution contains 1 mg/ mL of Lacosamide.

4.2.3.4.5 Preparation of Calibration graph

In this method, the aliquots of 0.7 – 1.3 mL of standard stock solution of Lacosamide were transferred into a series of 10 mL volumetric flasks and made up to the mark with mobile phase. The solutions containing the concentrations of 70 - 130 µg/ mL of Lacosamide. All the solutions were injected and the chromatograms were recorded at 210 nm. The above concentration range was found to be linear and obeys Beer's law. The procedure was repeated for three times. The peak areas were plotted against concentration and the calibration curve was constructed.

4.2.3.4.6 Estimation of Lacosamide in tablet formulation

Ten tablets (LACOSAM) tablets were weighed accurately. The average weight was found and powdered. The tablet powder equivalent to 100 mg of

Lacosamide was weighed and transferred into 100 mL volumetric flask and added a minimum quantity of methanol (HPLC grade) to dissolve the substance and the solution was sonicated for 15 minutes. The solution was made up to 100 mL with methanol. The solution was centrifuged at 2000 rpm for 15 minutes and filtered through Wattmann filter Paper No: 41.

4.2.3.4.6.1 Assay Procedure

8, 10, 12 mL of test solution was transferred into nine 100 mL volumetric flasks (three for each concentration i.e., Low level, Mid level and High level) and made up to the mark with mobile phase. A steady base line was recorded with optimized chromatographic conditions. After the stabilization of base line for 30 minutes, nine test solutions of formulation were injected and recorded the chromatograms. The concentration of each test solution was determined by using peak area values from the calibration graph.

4.2.3.4.7 Recovery Experiments

4.2.3.4.7.1 Preparation Lacosamide raw material stock solution

An accurately weighed quantity of 400 mg of Lacosamide was transferred into 10 mL volumetric flask and added sufficient methanol (HPLC grade) to dissolve the substance and made up to the mark with the same. This contains 40 mg/ mL concentration.

4.2.3.4.7.2 Procedure

The recovery experiment was done by adding known concentrations of raw material stock solution of Lacosamide to the preanalyzed formulation. The tablet powder equivalent to 100 mg of Lacosamide was weighed accurately and added 2mL, 2.5 mL, and 3 mL of raw material stock solution into the same 100 mL standard flasks individually and dissolved in methanol and the solution was sonicated for 15 minutes.

The solution was made up to 100 mL with methanol. The solution was centrifuged for 15 minutes at 2000 rpm and the solution was filtered through Whatmann filter Paper No: 41 and made up to volume with mobile phase. The procedure was repeated as per the analysis of formulation. The amount of drug recovered was calculated by using slope and intercept valves from the calibration graph.

4.2.3.4.8 Validation

4.2.3.4.8.1 Linearity

A calibration curve was plotted between concentration and peak area. Lacosamide was linear in the concentration range of 70 – 130 μg/ mL of Lacosamide.

4.2.3.4.8.2 Precision

The repeatability of the method was confirmed by the analysis of formulation was repeated for six times with the same concentration. The amount of drug present in the tablet formulation was calculated. The percentage RSD was calculated. The intermediate precision of the method was confirmed by intraday and interday analysis i.e. the analysis of formulation was repeated three times in the same day and on three successive days. The amount of drugs was determined and the percentage RSD also calculated.

4.2.3.4.8.3 Accuracy (Recovery studies)

Accuracy of the method was confirmed by recovery studies. To the preanalyzed formulation, a known quantity of raw material of Lacosamide was added and the procedure was followed as per the analysis of formulation. The amount of each drug recovered was calculated. This procedure was repeated for three times for each concentration. The % RSD was calculated.

4.2.3.4.8.4. LOD and LOQ

The linearity study was carried out for six times. The LOD and LOQ were calculated based upon the calibration curve method. The LOD and LOQ were calculated by using the average of peak area and standard deviation of intercept.

4.2.4 HPTLC Method

In HPTLC, the separation of the components of a mixture is based upon the principle of adsorption. HPTLC is differing from the TLC in the size of the silica gel used as the stationary phase, automated sampling application and detection. In the present study, a twin trough chamber and silica gel 60 F 254 plates were used.

4.2.4.1 Optimization of chromatographic method

The initial separation conditions used for the separation of Lacosamide are as follows.

Stationary Phase	-	silica gel 60 F 254 on Aluminium sheet
Mobile Phase	-	Methanol: Ammonia
Ratio	-	5:4 % v/v
Detection	-	UV light
Temperature	-	30° C
Chamber	-	Twin Trough Chamber
Development Mode	-	Ascending Mode

The mobile phase was allowed to saturate for 20 minutes in the chamber. 1 µl volume of 1mg/ mL solution was spotted on the plates and developed the chromatograms.

4.2.4.1.1. Selection of Mobile Phase

Different mixtures of mobile phase were tried to choose the best mobile phase for the analysis. They include the following

Methanol: Ammonia (5:4 % v/v/)

Methanol: Water (5:5 % v/v)

Chloroform: Water (4: 6% v/v)

Acetonitrile: Water (4: 6 % v/v)

From the above list of mobile phase the mobile phase Acetonitrile: Water (8:2 % v/v/) was found to be a better mobile phase. The drug was eluted with good peak.

4.2.4.1.2. Selection of detection wavelength

The spectrum of Lacosamide was recorded in the selected mobile phase. 257 nm was selected as detection wave length where the absorbance was maximum.

4.2.4.1.3. Optimized Chromatographic Conditions

After conforming with the mobile phase and the detection wavelength, the optimized conditions for the method was as follows,

Stationary Phase	-	Silica Gel 60 F 254 HPTLC Plates
Mobile Phase	-	Acetonitrile: Water
Ratio	-	(8:2 % v/v/)
Detection	-	CAMAG TLC Scanner 3, at 257 nm
Temperature	-	Ambient
Chamber	-	Twin Trough Chamber
Development Mode	-	Ascending Mode

4.2.4.2. Preparation of standard stock solution

10 mg of Lacosamide was weighed accurately and transferred into a 10 mL volumetric flask. Dissolved in methanol and the volume was made up to 10 mL with methanol to get a concentration of 1mg / mL.

4.2.4.3. Linearity and Calibration Curve

Form the standard solution 1 – 6 µL were spotted at regular intervals in the plate to get a concentration range of 1to 6 µg/ µl of Lacosamide. The plates were developed and the calibration graph was plotted using peak area versus concentration.

4.2.4.4. Quantification of formulation

Twenty tablets were weighed accurately and the average weight of each tablet was determined. The tablets were crushed into fine powder. The tablet powder equivalent to 50 mg of Lacosamide was weighed in to a 50 mL volumetric flask, dissolved in methanol, sonicated for 15 minutes and made up to 50 mL with methanol. This solution was centrifuged for 15 minutes at 2000 rpm. The solution was filtered through a Whatmann filter paper No. 41. Six 3 µl spots were placed on the plates and the chromatograms were recorded. From the peak area the amount of the drug was calculated.

4.2.4.5. Recovery studies

4.2.4.5.1. Preparation of Lacosamide raw material stock solution

40 mg of Lacosamide raw material was weighed in to a 10 mL volumetric flask, dissolved well with methanol and made up to 10 mL with methanol to get the concentration of 4 mg/ mL.

4.2.4.5.2 Procedure

The recovery experiment was done by adding known concentrations of raw material stock solution of Lacosamide to the preanalyzed formulation. Tablet powder equivalent to 10 mg of Lacosamide was weighed in to three separate 10 mL volumetric flasks and dissolved in methanol, to this added 2 mL, 2.5 mL and 3 mL of Lacosamide raw material stock solution. Sonicated for 15 minutes and made up to 10 mL with methanol. This solution was centrifuged for 15 minutes at 2000 rpm. The solution was

filtered through a Whatmann filter paper No. 41. From the clear solution, 3 µl spot was placed from each flask and the chromatograms were recorded. The amount of drug recovered was calculated. The procedure was reported for three times.

4.2.4.6. Validation

4.2.4.6.1 Linearity

A calibration curve was plotted between concentration and peak area. Lacosamide was linear in the concentration range of 1-6 µg/ µL of Lacosamide.

4.2.4.6.2 Precision

The repeatability of the method was confirmed by the analysis of formulation was repeated for six times with the same concentration. The amount of drug present in the tablet formulation was calculated. The percentage RSD was calculated. The intermediate precision of the method was confirmed by intraday and interday analysis i.e. the analysis of formulation was repeated three times in the same day and on three successive days. The amount of drugs was determined and the percentage RSD also calculated.

4.2.4.6.3. Accuracy (Recovery studies)

Accuracy of the method was confirmed by recovery studies. To the preanalyzed formulation, a known quantity of raw material of Lacosamide was added and the procedure was followed as per the analysis of formulation. The amount of each drug recovered was calculated. This procedure was repeated for three times for each concentration. The % RSD was calculated.

4.2.4.6.4. LOD and LOQ

The linearity study was carried out for six times. The LOD and LOQ were calculated based upon the calibration curve method. The LOD and LOQ were calculated by using the average of slope and standard deviation of intercept.

RESULTS

AND

DISCUSSION

5. RESULTS AND DISCUSSION

Three methods were performed and developed for estimation of Lacosamide in bulk pure form and in tablet dosage form. The method are employed for analysis of Lacosamide were

5.1 First order Derivative spectrophotometric method

5.2RP-HPLC method

5.3HPTLC method

5.1 First order Derivative Spectrophotometric method

The identification of Lacosamide was confirmed by melting point analysis and IR spectral studies as shown in figure 1 and the solubility profile for Lacosamide was tried with various solvents (as in table 1) and Distilled water was selected as solvent. The zero order spectrums were derivatized into first order derivative spectrum. The first order derivative spectrum of Lacosamide was recorded as shown in Figure 2. From the spectrum, 216.5 nm was selected for the estimation of Lacosamide, which has maximum absorbance at this wavelength. Aliquots of Lacosamide were prepared in the concentration range of 10 - 50 µg / mL. The $\Delta a/\Delta\lambda$ value of these solutions was measured at 216.5 nm in the first order derivative spectrum for Lacosamide. The plotted calibration curve was shown in Figure 3. The preparation of calibration curve was repeated for six times at their selective wavelength. The calibration curve was plotted using concentration against $\Delta a/\Delta\lambda$. The optical parameters like Sandell's sensitivity, Molar absorptivity, correlation coefficient, slope, intercept, LOD, LOQ and Standard error were calculated. The correlation coefficient of the drug was found to be above 0.9998. This indicates that the drug obeys Beer's law and it was linear at the selected concentration range. The results are shown in Table 2.

For Quantification, the concentration of solution containing 30µg/ mL of Lacosamide was selected, prepared and measured at the selected wavelength. The amount of six test solutions was determined and the percentage purity of tablets was found to be 99.55 ± 1.1795 for Lacosamide. The amount present in tablets was in good concord with the label claim and the % RSD values were found to be 1.1848 for Lacosamide. The results of analysis are shown in Table 3. The low % RSD values indicate that the method has good precision.

Further, the precision of the method was confirmed by Intraday and Interday analysis. The analysis of tablets was carried out for three times in the same day and one time in the three consecutive days. The % RSD value for Intraday and Interday analysis were 0.4124 and 0.1986 for Lacosamide, respectively. The results of analysis are shown in Table 4 and 5. Hence the precision was confirmed.

The developed method was validated for Ruggedness. In the present work, ruggedness was confirmed by different analysts. The % RSD value by analyst 1 and analyst 2 were found to be 0.1243 and 0.0813 for Lacosamide, respectively. By instrument 1 and instrument 2 were found to be 0.1118 and 0.0905, respectively. The low % RSD value indicates that the developed method was more rugged. The results are shown in Table 6 and 7.

The accuracy of the method was performed by recovery studies. To the preanalysed formulation, a known quantity of Lacosamide raw material solutions was added at three different concentration levels. The absorbance of the solutions was measured and the percentage recovery was calculated. The percentage recovery was found to be in the range of 99.18 – 99.89 %. The average % RSD value of was found to be 0.5069. The low % RSD values of the drug reveals that the method was more accurate. The recovery data was shown in Table 8.

5.2 RP - HPLC Method

An exertion has been made to simple, precise, rapid, specific and accurate method for the estimation of Lacosamide in pure form and in formulation by RP – HPLC method.

The solution of 10 µg/ mL of Lacosamide in mobile phase was prepared and the solution was scanned in the range of 200 – 400 nm. At 210 nm the drug showed maximum absorbance and it was selected as detection wavelength for estimation of Lacosamide by RP – HPLC method with isocratic elution technique and it was found to be stable up to 3 hours 30 minutes.

Based up on the properties of the drug the initial separation was achieved by using different mobile phase with different compositions and the tried the mobile phase consists of 50Mm Potassium Dihydrogen Orthophosphate: Acetonitrile: Methanol (50: 40: 10 % v/v/v, 30: 20: 50 % v/v/ v and 50: 20: 30 % v/ v/ v) was initially tried and chromatograms were recorded.

At 50: 40: 10 % v/v ratio, the drug was eluted with broad, splitted peak and at 30: 20: 50 % v/v the peak for Lacosamide was observed with tailing and in 50Mm Potassium dihydrogen Orthophosphate : Acetonitrile: Methanol (50: 20: 30 % v/v/v) composition a sharp peak with slight tailing was observed. This is shown in figure 4, 5 and 6 respectively.

Finally for HPLC Analysis the selected mobile phase was 50 Mm Potassium dihydrogen orthophosphate :Acetonitrile: Methanol in the ratio of 30: 10: 60 % v/v/v with a retention time of 2.697 minutes as shown in figure 7.The system suitability parameters for optimized chromatogram are shown in table 9.

With the optimized chromatographic conditions, stock solutions of Lacosamide were prepared by using methanol (for first dilution only) and mobile

phase in the concentrations in the range of 70 -130 µg/ mL. The chromatograms are shown in the figure 8 - 14

The calibration curve was plotted using concentration against peak area. The procedure was repeated for three times. The correlation co-efficient value was found to be 0.9997. The calibration graph is shown in figure 15. The optical characteristics like correlation coefficient, slope, intercept, LOD, LOQ were calculated and are shown in table 10.

The tablet formulation (LACOSAM) was selected for the analysis. The nominal concentration (100 µg/ mL) from calibration curve was prepared in mobile phase. 20 µl quantity of formulation was injected and the chromatogram was recorded. The chromatograms are shown in figures 16 - 24. The percentage purity of Lacosamide present in formulation was found to be 99.47% ± 0.3760. The % RSD value was found to be 0.3768 and it indicates that the method has good precision. The values are shown in table 11.

The accuracy of the method was performed by recovery studies. To the preanalyzed formulation a known quantity of Lacosamide raw material solution was added at three different concentrations (80%, 100% and 120%) and solution were injected. The chromatograms were recorded and are shown in the figures 25 -27. The percentage recovery was found to be in the range of 99.59 % to 100.41% of Lacosamide. The % RSD values were found to be 0.4455. The Low % RSD values indicated that there are no interferences due to the excipients used in formulation during the analysis of Lacosamide from tablet formulation. Hence the method was found to be accurate. The recovery data is shown in table 12.

The system suitability parameters and all the validation parameters were found to be within the limit. Hence, this method can be effectively applied for the

estimation of Lacosamide in pure and in pharmaceutical dosage form than the reported methods.

5.3 HPTLC METHOD

The initial separation was made using various mobile phase using chloroform, methanol, formic acid, acetic acid, ammonia and water in different combinations. The mobile phases tried were Methanol: Ammonia (6: 4 % v/v), chloroform: Water (4:6). Methanol: Water (5: 5 % v/v) and Acetonitrile: water (4:6 % v/v) were tried.

In Methanol: Ammonia (6:4 % v/v) the drug was separated and the R_f value found to be 0.90 The R_f value of Lacosamide was beyond the normal values (range from 0.2 to 0.8), (P.D Sethi et al). Hence the different ratios were tried with the same mobile phase. Acetonitrile: Water in the ratio of 8:2 % v/v was selected for the method since, the drug was eluted with good resolution. 257 nm was selected as the detection wavelength for the analysis. The spectral conformation of the standard Lacosamide with sample solution Lacosamide is given in figure 28.

1 mg/ mL stock solution of Lacosamide was prepared in methanol. From the stock solution concentration range of 1µg to 6µg/ spot of Lacosamide chromatogram were developed in the Twin trough Chamber. The linearity chromatograms are shown in figure 29- 34. The calibration graph was plotted with concentration versus peak area and the correlation coefficient was found to be 0.9992 for Lacosamide. The calibration graph for Lacosamide was shown in figure 35. The optical characteristics such as the LOD, LOQ, Slope, Intercept, Regression equation and correlation coefficient are given in table 13.

The method was applied for the analysis of formulation. The percentage purity of Lacosamide was found to be 99.13% ± 1.1737. The % RSD values for Lacosamide 1.1840. The precision of the method was confirmed by the repeatable analysis of

formulation for six times. The chromatograms are given in figures 36 - 41. The data is given in table 14.

The intermediate precision of the method was confirmed by Intraday and Inter day analysis. The analysis of formulation was repeated for three times in the same day and one time in the three successive days. The percentage RSD value for intraday and inter day analysis of Lacosamide was found to be 0.3502 and 1.4218, respectively. The reports of analysis are shown in tables 15 and 16, respectively. The results showed that the precision of the method was further confirmed.

The accuracy of the method is confirmed by the recovery analysis. To the pre-analyzed formulation, known quantities of the standard drugs were added at three different concentrations. The amount of Lacosamide recovered was in the range of 99.09 % to 102.36%. The %RSD values were found to be 1.6790. The low % RSD values indicate that there is no interference of the excipients during the analysis. The peaks of the developed chromatograms are given in figures 42 - 44.The data of recovery analysis are given in table 17.

SUMMARY AND CONCLUSION

6. SUMMARY AND CONCLUSION

Three methods were performed and developed for estimation of Lacosamide in bulk pure form and in tablet dosage form. The method are employed for analysis of Lacosamide were

6.1 First order Derivative spectrophotometric method

6.2 RP-HPLC method

6.3 HPTLC method

6.1 First Order Derivative Spectroscopy

From the solubility profile Distilled water was chosen as a solvent for the estimation of Lacosamide. The sample solution of 10 μg/ mL of Lacosamide in Distilled water was prepared and the solution was scanned in UV region in the wavelength range from 200 to 400 nm by using methanol as blank. From the spectra, Lacosamide shows maximum absorbance at 216.5 nm.

The percentage label claim present in capsule formulation was found to be 99.55 ± 1.1795%. The percentage recovery was found to be in the range of 99.18 – 99.89 %.

6.2 RP-HPLC METHOD

An exertion has been made for a simple, precise, rapid, specific and accurate method for the estimation of Lacosamide in pure form and in formulation by RP – HPLC method.

After optimizing the mobile phase, with the consideration of the system suitability parameters, 50 mM Potassium Dihydrogen Orthophosphate: Acetonitrile: Methanol in the ratio of 50: 20: 30 % v/v was selected as mobile phase for the analysis.

With the optimized chromatographic conditions, the drug was linear in the concentration range of 70 - 130 μg/ ml. The correlation coefficient was found to be 0.9993.

The percentage purity of Lacosamide present in formulation was found to be 99.74 ± 0.3760. The precision of the method was confirmed by repeatability of formulation for six times.

The percentage recovery was found to be in the range of 99.59 – 100.41.

6.3 HPTLC METHOD

A simple and rapid HPTLC method was developed for Lacosamide in bulk and in tablet dosage form. The mobile phase consisting of Acetonitrile: water in the ratio of 8:2 % v/v was selected for the analysis. From the spectral characteristics, 257 nm was selected as the detection wavelength for the analysis. With the optimised conditions, the linearity range was fixed as 1 to 6 μg/ μl for Lacosamide. The correlation coefficient for the Lacosamide was found to be 0.9992.

LACOSAM tablets were selected for analysis. The percentage label claim present in the tablet formulation was found to be 99.13 ± 1.1737 of Lacosamide. The precision of the method was confirmed by the reported analysis of formulation. The %RSD was found to be 1.1840.

Further precision of the method was confirmed by Intraday and Inter day analysis. The percentage RSD value of the intraday and inter day analysis of Lacosamide was found to be 0.3502 and 1.4218 respectively.

The accuracy of the method was confirmed by recovery studies. The percentage recovery was found to be in the range of 99.09 % to 102.36% of Lacosamide.

Three simple, rapid and accurate methods like First order derivative spectrophotometric method, isocratic RP – HPLC method and HPTLC method were developed for the determination of Lacosamide in bulk and in tablet formulation. The methods showed excellent sensitivity, reproducibility, accuracy and repeatability, which is evidenced by low percentage relative standard deviation values. The results obtained in recovery studies were indicating that there is no interference from the excipients used in the formulation. By comparing three methods, UV spectroscopic method was found to be economic when compared to RP – HPLC and HPTLC. Because the solvents and column used in RP – HPLC and HPTLC are very costly. When comparing the sensitivity of the methods, RP – HPLC and HPTLC methods were found to be more sensitive than UV spectroscopic method. Because the linearity range, LOD and LOQ were less. Hence it is suggested that the proposed UV spectroscopic method, HPTLC method and RP - HPLC method can be effectively applied for the routine analysis of Lacosamide in bulk and in tablet formulation.

FIGURES

FIGURE-2

FIRST ORDER DERIVATIVE SPECTRUM OF LACOSAMIDE

IN DISTILLED WATER

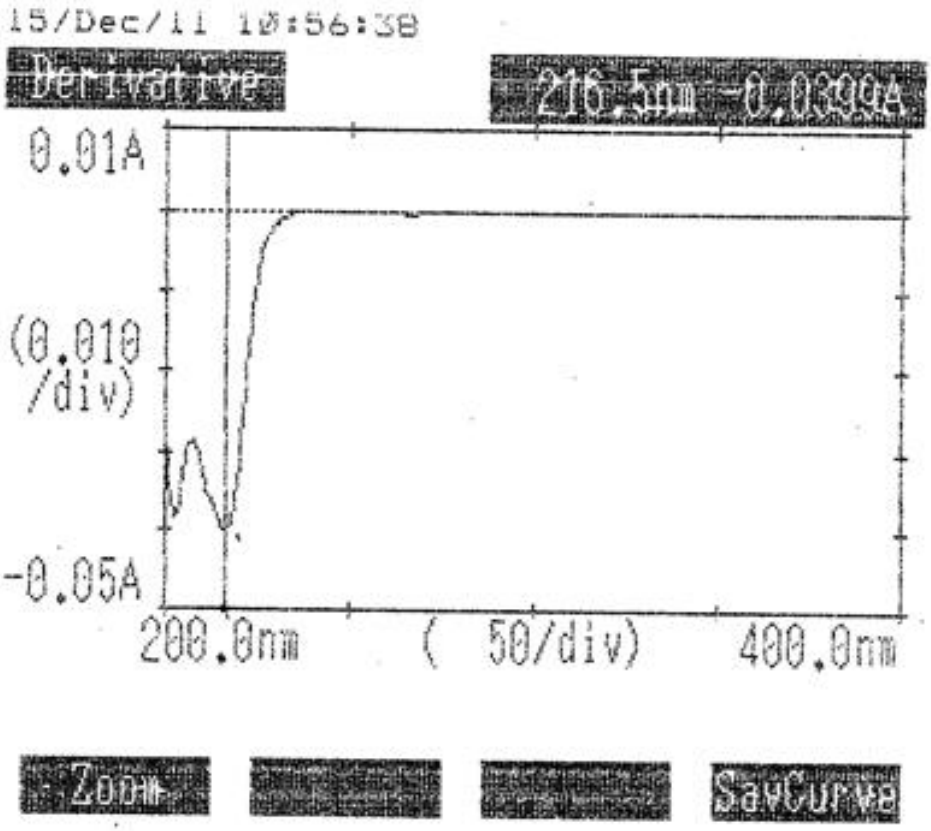


FIGURE-3

CALIBRATION CURVE OF LACOSAMIDE BY FIRST ORDER DERIVATIVE SPECTROPHOTOMETRIC METHOD USING DISTILLED WATER

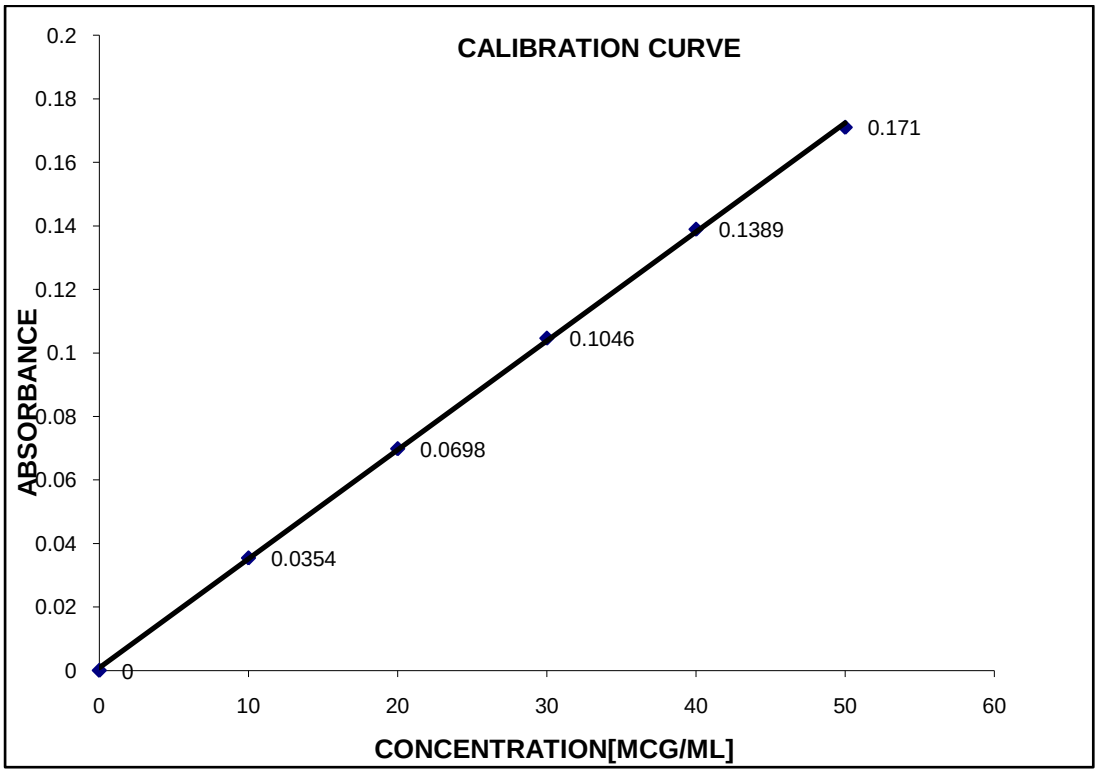
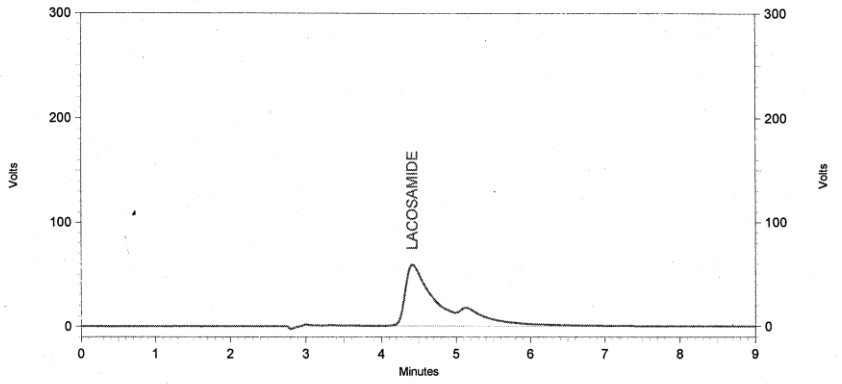


FIGURE-4

CHROMATOGRAM FOR THE EFFECT OF RATIO OF MOBILE PHASE OF LACOSAMIDE 50Mm POTASSIUM DIHYDROGEN ORTHRO PHOSPHATE: ACETONITRILE: METHANOL (50:40:10)

Area % Report

Description: LACOSAMIDE – METHOD DEVELOPEMENT
Data File: D:\ChromQuest\PROJECTS\DATA\LACOSAMIDE\FEB 2012\18.02.2012\TRIAL 01_.dat
Method: D:\ChromQuest\Method\LACOSAMIDE FP.met
Acquired: 2/18/2012 4:25:05 PM
Wave Length: 210 nm



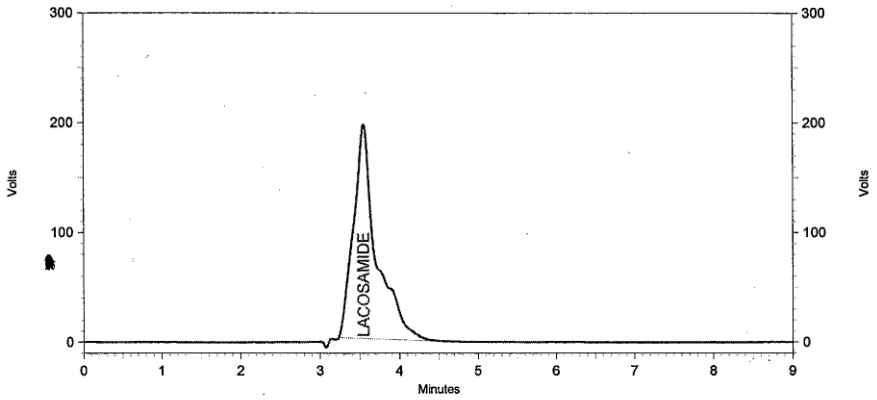
Name	Retention Time	Area	Area %	Height	Height Percent	Asymmetry	Theoretical plates (USP)	Capacity factor
LACOSAMIDE	4.418	1972366	100.00	58598	100.00	4.03	673	3.4
Totals		1972366	100.00	58598	100.00			

FIGURE-5

CHROMATOGRAM FOR THE EFFFECT OF RATIO OF MOBILE PHASE OF LACOSAMIDE 50Mm POTASSIUM DIHYDROGEN ORTHRO PHOSPHATE: ACETONITRILE: METHANOL (30:20:50)

Area % Report

Description: LACOSAMIDE – METHOD DEVELOPEMENT
Data File: D:\ChromQuest\PROJECTS\DATA\LACOSAMIDE\FEB 2012\18.02.2012\TRIAL 02_.dat
Method: D:\ChromQuest\Method\LACOSAMIDE FP.met
Acquired: 2/18/2012 5:13:01 PM
Wave Length: 210 nm



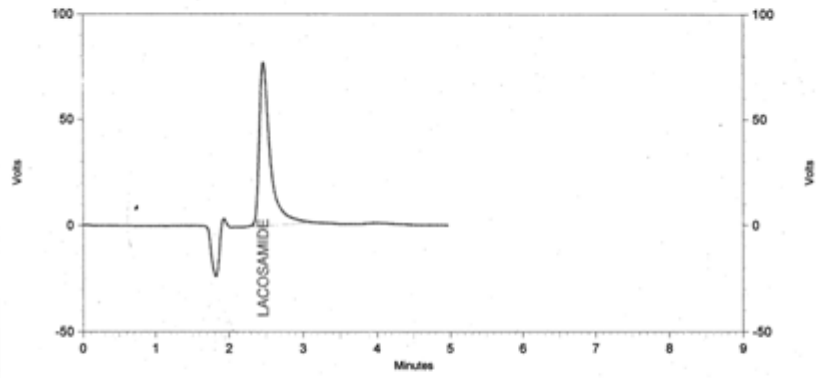
Name	Retention Time	Area	Area %	Height	Height Percent	Asymmetry	Theoretical plates (USP)	Capacity factor
LACOSAMIDE	3.550	3899305	100.00	194933	100.00	1.53	1052	2.6
Totals		3899305	100.00	194933	100.00			

FIGURE-6

CHROMATOGRAM FOR THE EFFFECT OF RATIO OF MOBILE PHASE OF LACOSAMIDE 50Mm POTASSIUM DIHYDROGEN ORTHRO PHOSPHATE: ACETONITRILE: METHANOL (50:20:30)

Area % Report

Description: LACOSAMIDE – METHOD DEVELOPEMENT
Data File: D:\ChromQuest\PROJECTS\DATA\LACOSAMIDE\FEB 2012\18.02.2012\TRIAL 03_.dat
Method: D:\ChromQuest\Method\LACOSAMIDE FP.met
Acquired: 2/18/2012 6:33:01 PM
Wave Length: 210 nm

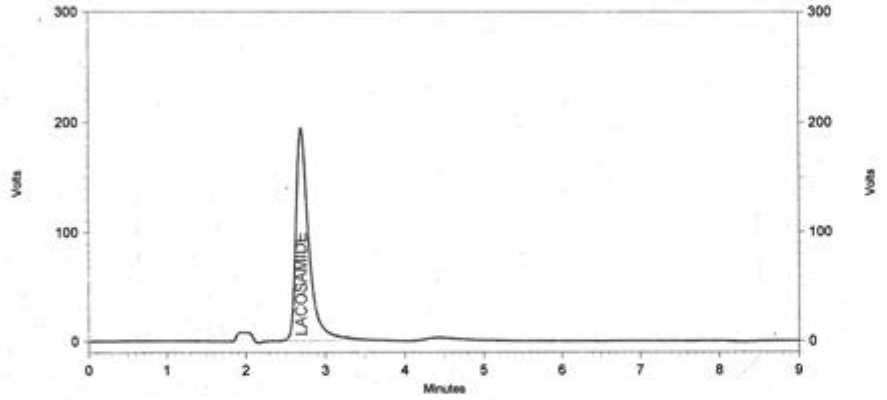


Name	Retention Time	Area	Area %	Height	Height Percent	Asymmetry	Theoretical plates (USP)	Capacity factor
LACOSAMIDE	2.468	797464	100.00	77116	100.00	2.05	1655	1.5
Totals		797464	100.00	77116	100.00			

FIGURE-7
OPTIMIZED CHROMATOGRAM OF LACOSAMIDE

Area % Report

Description: STD - LACOSAMIDE (100mcg/mL)
Data File: D:\ChromQuest\PROJECTS\DATA\LACOSAMIDE\FEB 2012\19.02.2012\A03_SS-02.dat
Method: D:\ChromQuest\Method\LACOSAMIDE FP.met
Acquired: 2/19/2012 1:54:17 AM
Wave Length: 210 nm



Name	Retention Time	Area	Area %	Height	Height Percent	Asymmetry	Theoretical plates (USP)	Capacity factor
LACOSAMIDE	2.690	2171186	100.00	193915	100.00	1.49	1545	1.7
Totals		2171186	100.00	193915	100.00			

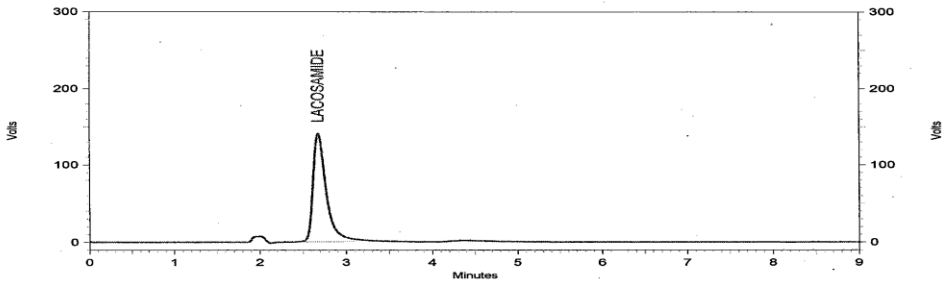
FIGURE-8

LINEARITY CHROMATOGRAM OF LACOSAMIDE

BY RP-HPLC (70 µg/ mL)

Area % Report

Description: STD - LACOSAMIDE (70mcg/mL)
Data File: D:\ChromQuest\PROJECTS\DATA\LACOSAMIDE\FEB 2012\19.02.2012\A007_LINERTY_70%.dat
Method: D:\ChromQuest\Method\LACOSAMIDE FP.met
Acquired: 2/19/2012 3:00:41 AM
Wave Length: 210 nm



Name	Retention Time	Area	Area %	Height	Height Percent	Asymmetry	Theoretical plates (USP)	Capacity factor
LACOSAMIDE	2.682	1505824	100.00	140486	100.00	1.46	1676	1.7
Totals		1505824	100.00	140486	100.00			

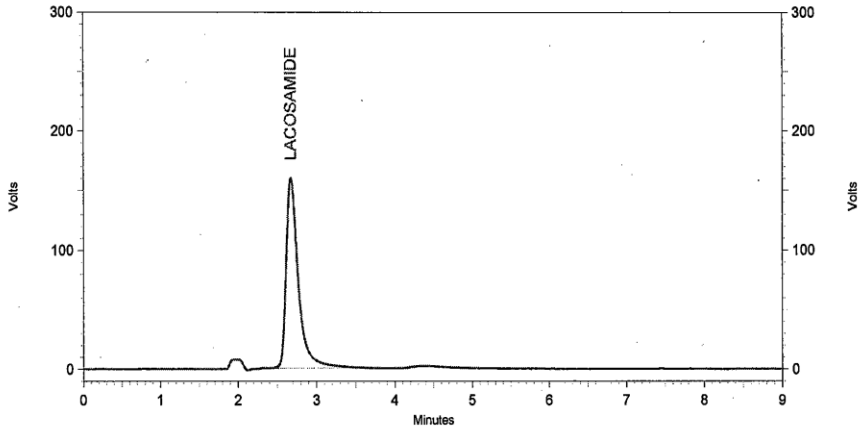
FIGURE-9

LINEARITY CHROMATOGRAM OF LACOSAMIDE

BY RP-HPLC (80 µg/ mL)

Area % Report

Description: STD - LACOSAMIDE (80mcg/mL)
Data File: D:\ChromQuest\PROJECTS\DATA\LACOSAMIDE\FEB 2012\19.02.2012\A008_LINERTY_80%.dat
Method: D:\ChromQuest\Method\LACOSAMIDE FP.met
Acquired: 2/19/2012 3:17:16 AM
Wave Length: 210 nm



Name	Retention Time	Area	Area %	Height	Height Percent	Asymmetry	Theoretical plates (USP)	Capacity factor
LACOSAMIDE	2.678	1721106	100.00	159567	100.00	1.45	1642	1.7
Totals		1721106	100.00	159567	100.00			

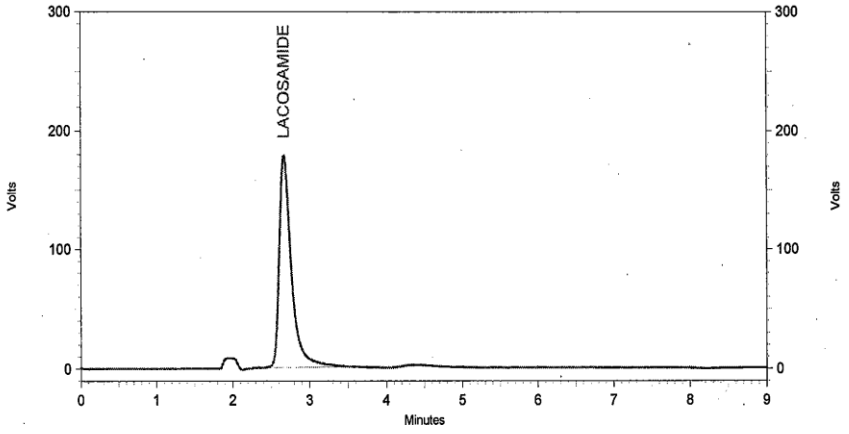
FIGURE-10

LINEARITY CHROMATOGRAM OF LACOSAMIDE

BY RP-HPLC (90 µg/ mL)

Area % Report

Description: STD - LACOSAMIDE (90mcg/mL)
Data File: D:\ChromQuest\PROJECTS\DATA\LACOSAMIDE\FEB 2012\19.02.2012\A009_LINERTY_90%.dat
Method: D:\ChromQuest\Method\LACOSAMIDE FP.met
Acquired: 2/19/2012 3:33:51 AM
Wave Length: 210 nm



Name	Retention Time	Area	Area %	Height	Height Percent	Asymmetry	Theoretical plates (USP)	Capacity factor
LACOSAMIDE	2.675	1950993	100.00	177985	100.00	1.43	1598	1.7

Totals								
		1950993	100.00	177985	100.00			

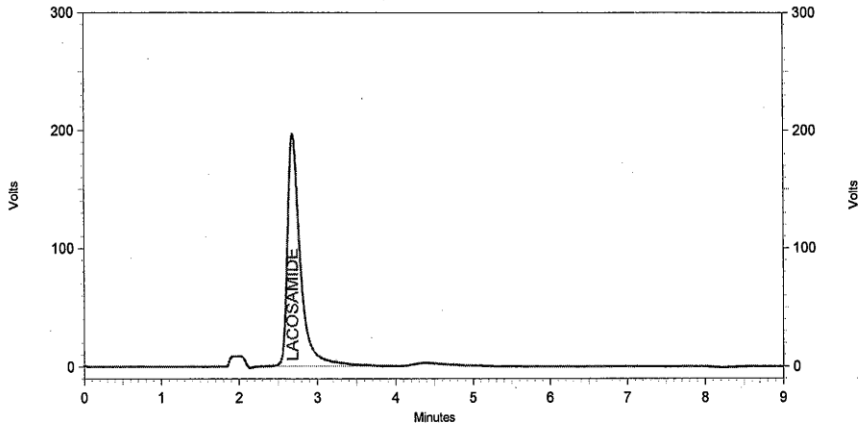
FIGURE-11

LINEARITY CHROMATOGRAM OF LACOSAMIDE

BY RP-HPLC (100 µg/ mL)

Area % Report

Description: STD - LACOSAMIDE (100mcg/mL)
Data File: D:\ChromQuest\PROJECTS\DATA\LACOSAMIDE\FEB 2012\19.02.2012\A010_LINERTY_100%.dat
Method: D:\ChromQuest\Method\LACOSAMIDE FP.met
Acquired: 2/19/2012 3:50:27 AM
Wave Length: 210 nm



Name	Retention Time	Area	Area %	Height	Height Percent	Asymmetry	Theoretical plates (USP)	Capacity factor
LACOSAMIDE	2.692	2200326	100.00	196253	100.00	1.44	1551	1.7

Totals								
		2200326	100.00	196253	100.00			

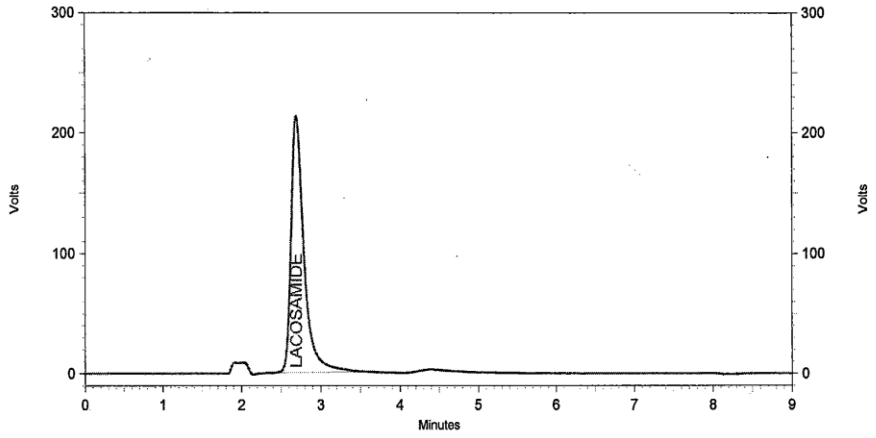
FIGURE-12

LINEARITY CHROMATOGRAM OF LACOSAMIDE

BY RP-HPLC (110 µg/ mL)

Area % Report

Description: STD - LACOSAMIDE (110mcg/mL)
Data File: D:\ChromQuest\PROJECTS\DATA\LACOSAMIDE\FEB 2012\19.02.2012\A011_LINERTY_110%.dat
Method: D:\ChromQuest\Method\LACOSAMIDE FP.met
Acquired: 2/19/2012 4:07:02 AM
Wave Length: 210 nm



Name	Retention Time	Area	Area %	Height	Height Percent	Asymmetry	Theoretical plates (USP)	Capacity factor
LACOSAMIDE	2.697	2409922	100.00	213187	100.00	1.48	1517	1.7
Totals		2409922	100.00	213187	100.00			

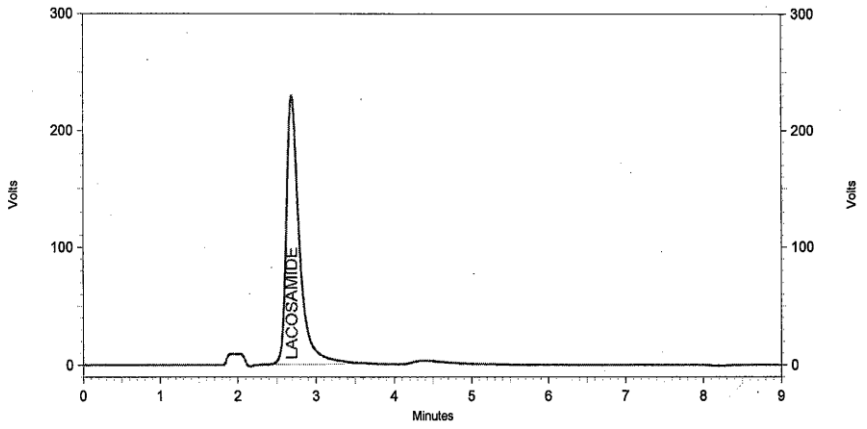
FIGURE-13

LINEARITY CHROMATOGRAM OF LACOSAMIDE

BY RP-HPLC (120 µg/ mL)

Area % Report

Description: STD - LACOSAMIDE (120mcg/mL)
Data File: D:\ChromQuest\PROJECTS\DATA\LACOSAMIDE\FEB 2012\19.02.2012\A012_LINERTY_120%.dat
Method: D:\ChromQuest\Method\LACOSAMIDE FP.met
Acquired: 2/19/2012 4:23:39 AM
Wave Length: 210 nm



Name	Retention Time	Area	Area %	Height	Height Percent	Asymmetry	Theoretical plates (USP)	Capacity factor
LACOSAMIDE	2.697	2655219	100.00	229347	100.00	1.45	1482	1.7

Totals								
		2655219	100.00	229347	100.00			

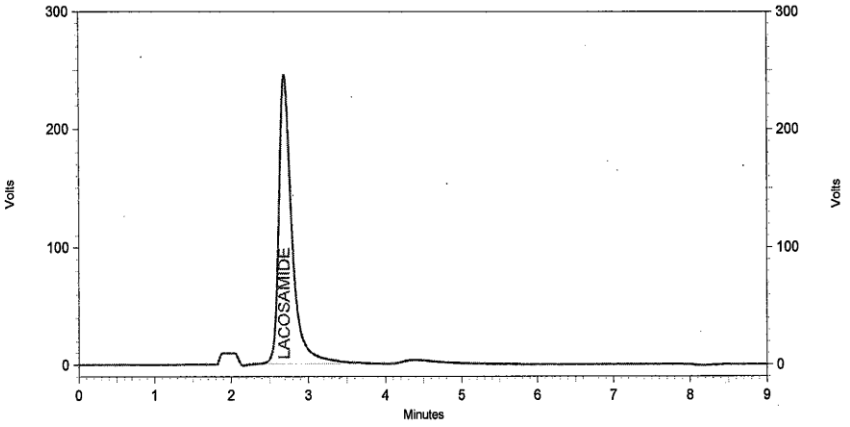
FIGURE-14

LINEARITY CHROMATOGRAM OF LACOSAMIDE

BY RP-HPLC (130 µg/ mL)

Area % Report

Description: STD - LACOSAMIDE (130mcg/mL)
Data File: D:\ChromQuest\PROJECTS\DATA\LACOSAMIDE\FEB 2012\19.02.2012\A013_LINERTY_130%.dat
Method: D:\ChromQuest\Method\LACOSAMIDE FP.met
Acquired: 2/19/2012 4:40:18 AM
Wave Length: 210 nm



Name	Retention Time	Area	Area %	Height	Height Percent	Asymmetry	Theoretical plates (USP)	Capacity factor
LACOSAMIDE	2.695	2892640	100.00	244844	100.00	1.43	1417	1.7

Totals								
		2892640	100.00	244844	100.00			

FIGURE-15

CALIBRATION CURVE OF LACOSAMIDE BY RP-HPLC

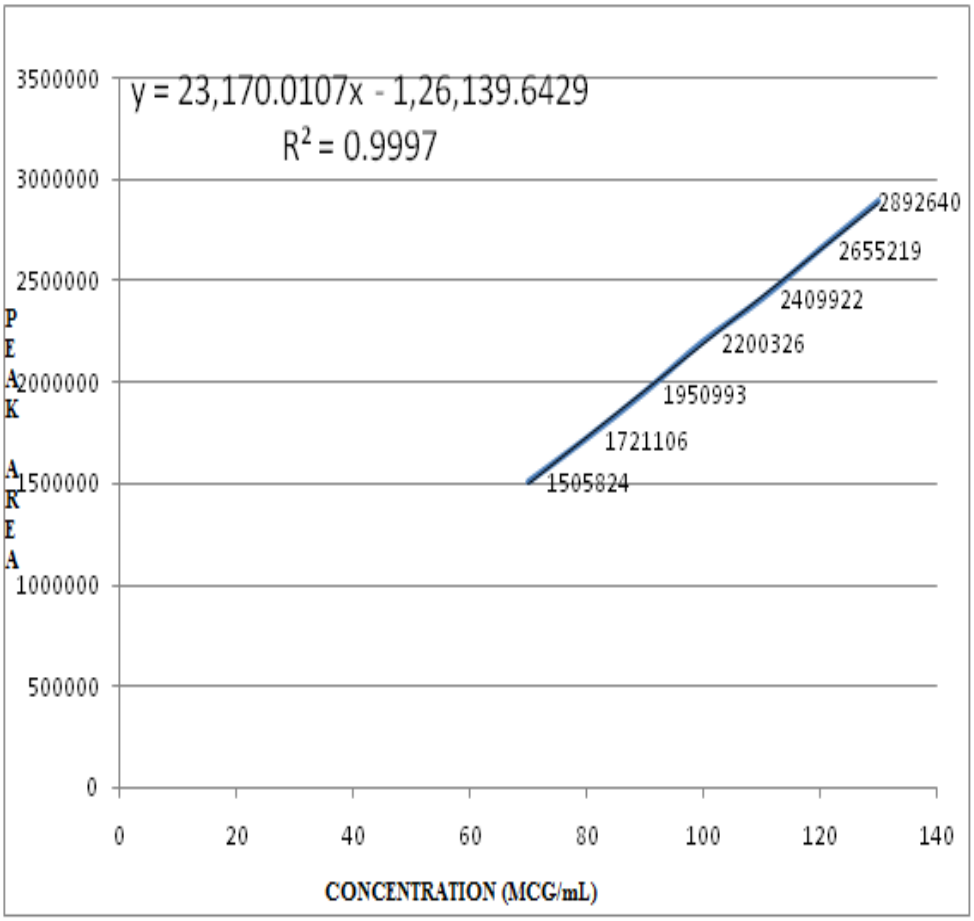
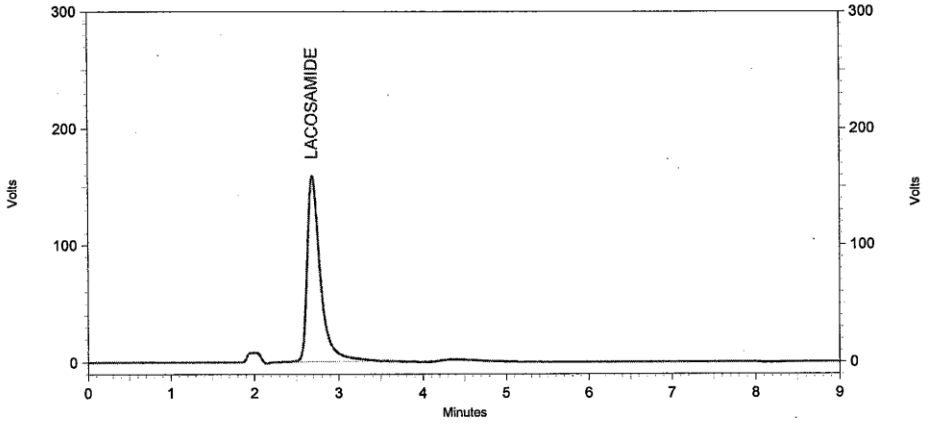


FIGURE-16

CHROMATOGRAM FOR ANALYSIS OF FORMULATION (LACOSAM) REPEATABILITY 1

Area % Report

Description: SPL- 01_LACOSAM-100_LOW LEVEL CONCENTRATION _(80mcg/mL)
Data File: D:\ChromQuest\PROJECTS\DATA\LACOSAMIDE\FEB 2012\19.02.2012\A014_P&A_L1.dat
Method: D:\ChromQuest\Method\LACOSAMIDE FP.met
Acquired: 2/19/2012 4:56:49 AM
Wave Length: 210 nm

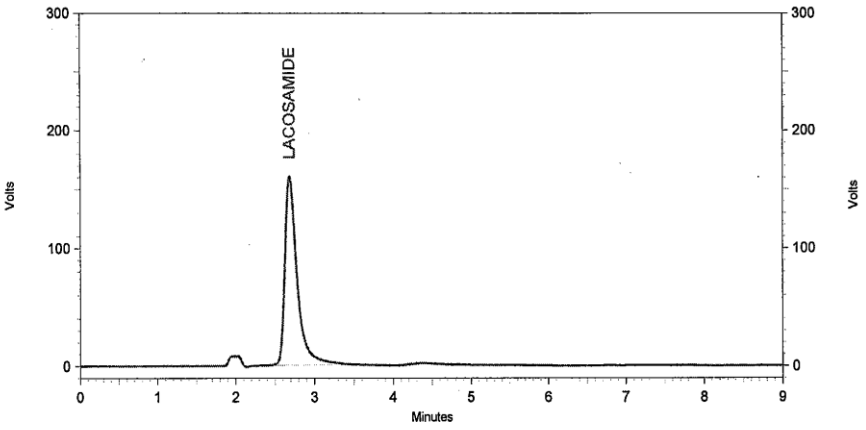


Name	Retention Time	Area	Area %	Height	Height Percent	Asymmetry	Theoretical plates (USP)	Capacity factor
LACOSAMIDE	2.695	1713490	100.00	158586	100.00	1.46	1668	1.7
Totals		1713490	100.00	158586	100.00			

FIGURE-17
CHROMATOGRAM FOR ANALYSIS OF FORMULATION (LACOSAM)
REPEATABILITY 2

Area % Report

Description: SPL- 02_LACOSAM-100_LOW LEVEL CONCENTRATION _(80mcg/mL)
Data File: D:\ChromQuest\PROJECTS\DATA\LACOSAMIDE\FEB 2012\19.02.2012\A015_P&A_L2.dat
Method: D:\ChromQuest\Method\LACOSAMIDE FP.met
Acquired: 2/19/2012 5:13:25 AM
Wave Length: 210 nm

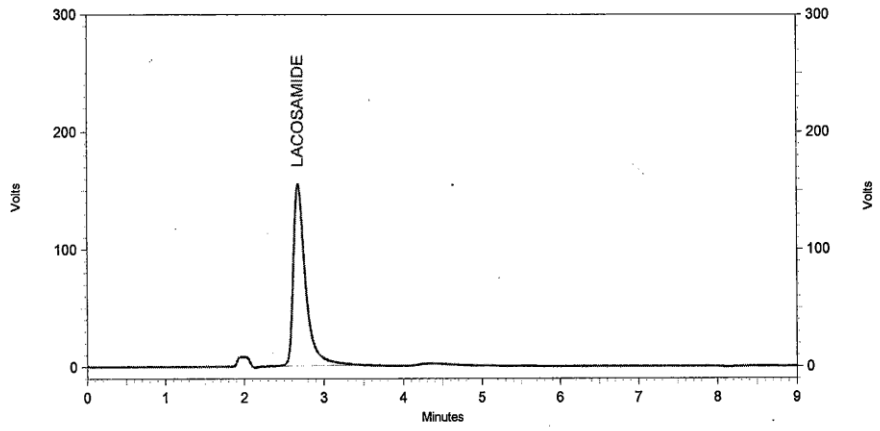


Name	Retention Time	Area	Area %	Height	Height Percent	Asymmetry	Theoretical plates (USP)	Capacity factor
LACOSAMIDE	2.690	1725717	100.00	159942	100.00	1.48	1672	1.7
Totals		1725717	100.00	159942	100.00			

FIGURE-18
CHROMATOGRAM FOR ANALYSIS OF FORMULATION (LACOSAM)
REPEATABILITY 3

Area % Report

Description: SPL- 03_LACOSAM-100_LOW LEVEL CONCENTRATION _(80mcg/mL)
Data File: D:\ChromQuest\PROJECTS\DATA\LACOSAMIDE\FEB 2012\19.02.2012\A016_P&A_L3.dat
Method: D:\ChromQuest\Method\LACOSAMIDE FP.met
Acquired: 2/19/2012 5:30:00 AM
Wave Length: 210 nm



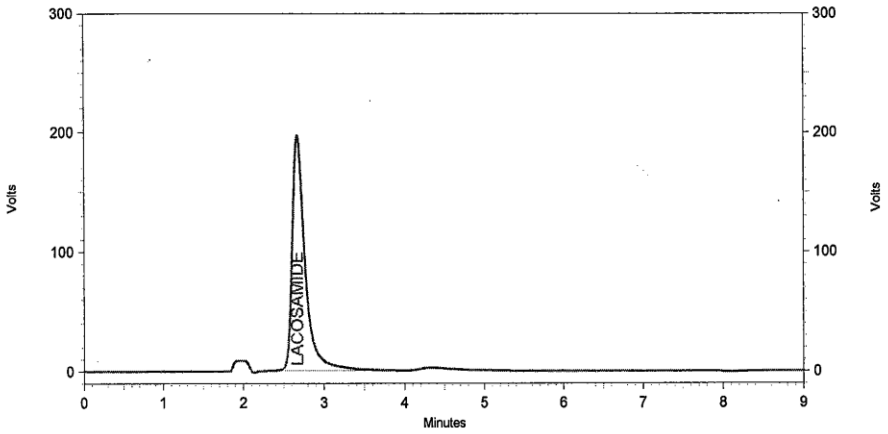
Name	Retention Time	Area	Area %	Height	Height Percent	Asymmetry	Theoretical plates (USP)	Capacity factor
LACOSAMIDE	2.683	1711461	100.00	154662	100.00	1.48	1691	1.7
Totals		1711461	100.00	154662	100.00			

FIGURE-19

CHROMATOGRAM FOR ANALYSIS OF FORMULATION (LACOSAM) REPEATABILITY 4

Area % Report

Description: SPL- 01_LACOSAM-100_MIDDLE LEVEL CONCENTRATION _(100mcg/mL)
Data File: D:\ChromQuest\PROJECTS\DATA\LACOSAMIDE\FEB 2012\19.02.2012\A017_P&A_M1.dat
Method: D:\ChromQuest\Method\LACOSAMIDE FP.met
Acquired: 2/19/2012 5:46:36 AM
Wave Length: 210 nm

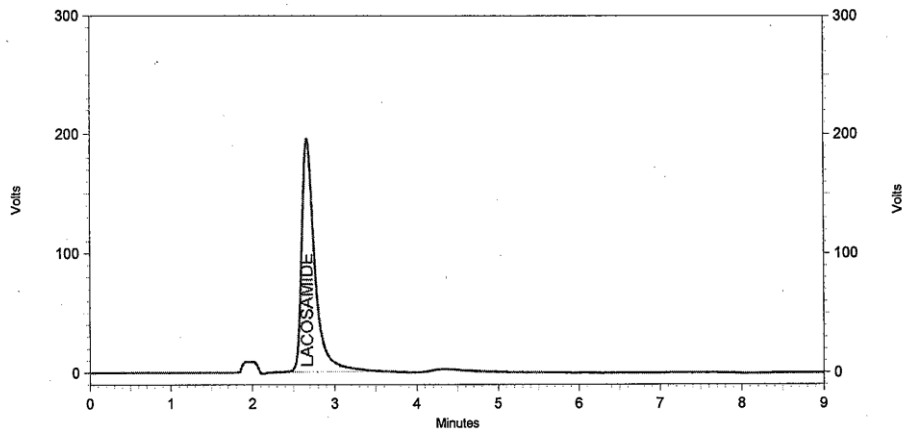


Name	Retention Time	Area	Area %	Height	Height Percent	Asymmetry	Theoretical plates (USP)	Capacity factor
LACOSAMIDE	2.677	2188626	100.00	196801	100.00	1.44	1590	1.7
Totals		2188626	100.00	196801	100.00			

FIGURE-20
CHROMATOGRAM FOR ANALYSIS OF FORMULATION (LACOSAM)
REPEATABILITY 5

Area % Report

Description: SPL- 02_LACOSAM-100_MIDDLE LEVEL CONCENTRATION _(100mcg/mL)
Data File: D:\ChromQuest\PROJECTS\DATA\LACOSAMIDE\FEB 2012\19.02.2012\A018_P&A_M2.dat
Method: D:\ChromQuest\Method\LACOSAMIDE FP.met
Acquired: 2/19/2012 6:03:07 AM
Wave Length: 210 nm



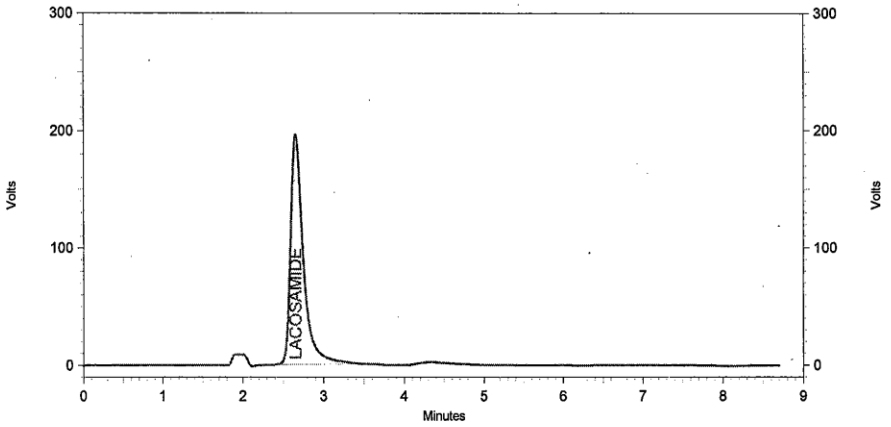
Name	Retention Time	Area	Area %	Height	Height Percent	Asymmetry	Theoretical plates (USP)	Capacity factor
LACOSAMIDE	2.672	2152106	100.00	195072	100.00	1.44	1590	1.7

Totals								
		2152106	100.00	195072	100.00			

FIGURE-21
CHROMATOGRAM FOR ANALYSIS OF FORMULATION (LACOSAM)
REPEATABILITY 6

Area % Report

Description: SPL- 03_LACOSAM-100_MIDDLE LEVEL CONCENTRATION _(100mcg/mL)
Data File: D:\ChromQuest\PROJECTS\DATA\LACOSAMIDE\FEB 2012\19.02.2012\A019_P&A_M3.dat
Method: D:\ChromQuest\Method\LACOSAMIDE FP.met
Acquired: 2/19/2012 6:14:53 AM
Wave Length: 210 nm



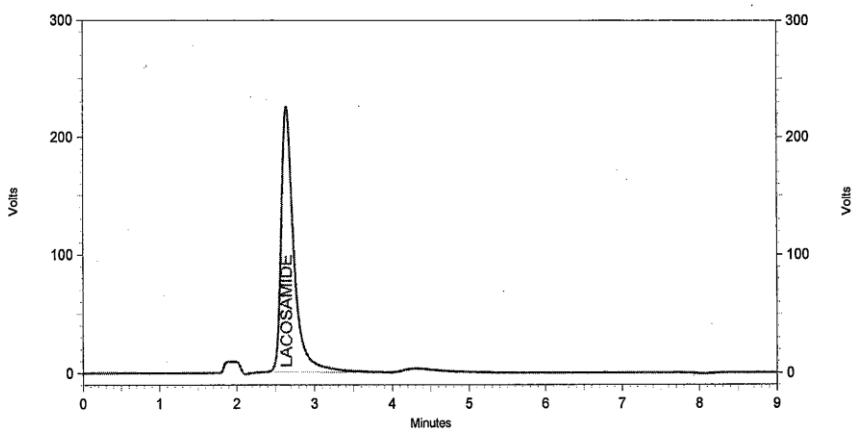
Name	Retention Time	Area	Area %	Height	Height Percent	Asymmetry	Theoretical plates (USP)	Capacity factor
LACOSAMIDE	2.658	2145953	100.00	195855	100.00	1.44	1579	1.7

Totals								
		2145953	100.00	195855	100.00			

FIGURE-22
CHROMATOGRAM FOR ANALYSIS OF FORMULATION (LACOSAM)
REPEATABILITY 7

Area % Report

Description: SPL- 01_LACOSAM-100_HIGH LEVEL CONCENTRATION _(120mcg/mL)
Data File: D:\ChromQuest\PROJECTS\DATA\LACOSAMIDE\FEB 2012\19.02.2012\A020_P&A_H1.dat
Method: D:\ChromQuest\Method\LACOSAMIDE FP.met
Acquired: 2/19/2012 6:24:58 AM
Wave Length: 210 nm



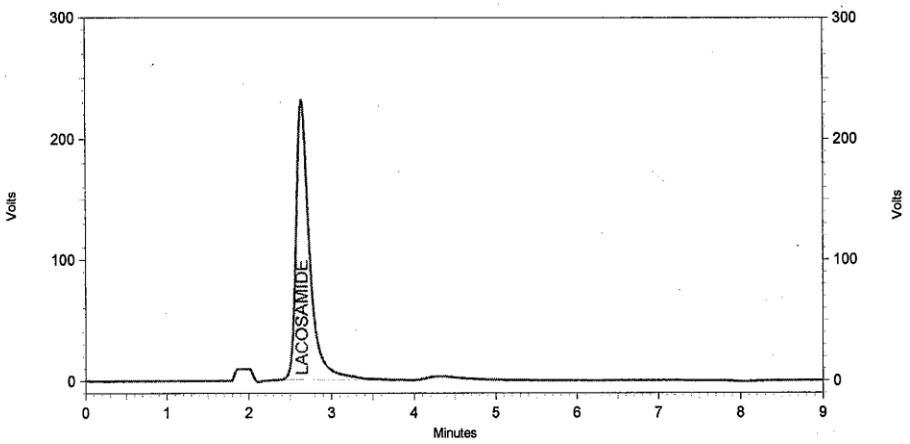
Name	Retention Time	Area	Area %	Height	Height Percent	Asymmetry	Theoretical plates (USP)	Capacity factor
LACOSAMIDE	2.647	2617706	100.00	225102	100.00	1.48	1496	1.7

Totals								
		2617706	100.00	225102	100.00			

FIGURE-23
CHROMATOGRAM FOR ANALYSIS OF FORMULATION (LACOSAM)
REPEATABILITY 8

Area % Report

Description: SPL- 02_LACOSAM-100_HIGH LEVEL CONCENTRATION _(120mcg/mL)
Data File: D:\ChromQuest\PROJECTS\DATA\LACOSAMIDE\FEB 2012\19.02.2012\A021_P&A_H2.dat
Method: D:\ChromQuest\Method\LACOSAMIDE FP.met
Acquired: 2/19/2012 6:35:49 AM
Wave Length: 210 nm

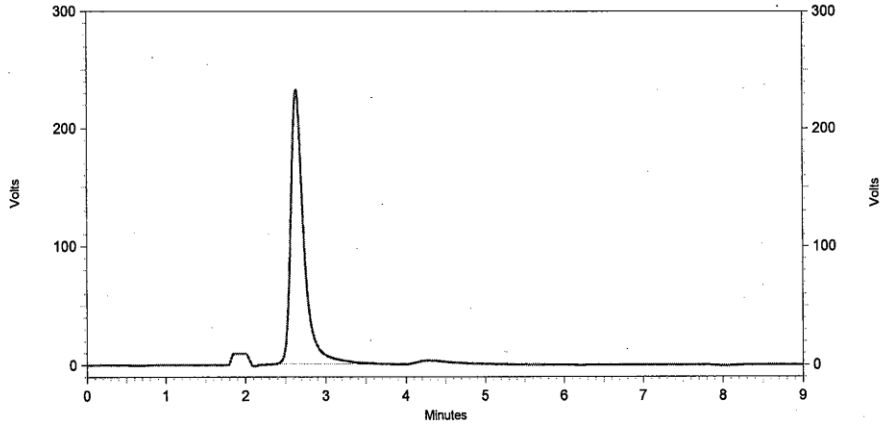


Name	Retention Time	Area	Area %	Height	Height Percent	Asymmetry	Theoretical plates (USP)	Capacity factor
LACOSAMIDE	2.647	2619065	100.00	230866	100.00	1.46	1481	1.7
Totals		2619065	100.00	230866	100.00			

FIGURE-24
CHROMATOGRAM FOR ANALYSIS OF FORMULATION (LACOSAM) REPEATABILITY 9

Area % Report

Description: SPL- 03_LACOSAM-100_HIGH LEVEL CONCENTRATION _(120mcg/mL)
Data File: D:\ChromQuest\PROJECTS\DATA\LACOSAMIDE\FEB 2012\19.02.2012\A022_P&A_H3.dat
Method: D:\ChromQuest\Method\LACOSAMIDE FP.met
Acquired: 2/19/2012 6:46:21 AM
Wave Length: 210 nm



Name	Retention Time	Area	Area %	Height	Height Percent	Asymmetry	Theoretical plates (USP)	Capacity factor
LACOSAMIDE	2.640	2622059	100.00	231735	100.00	1.45	1475	1.7

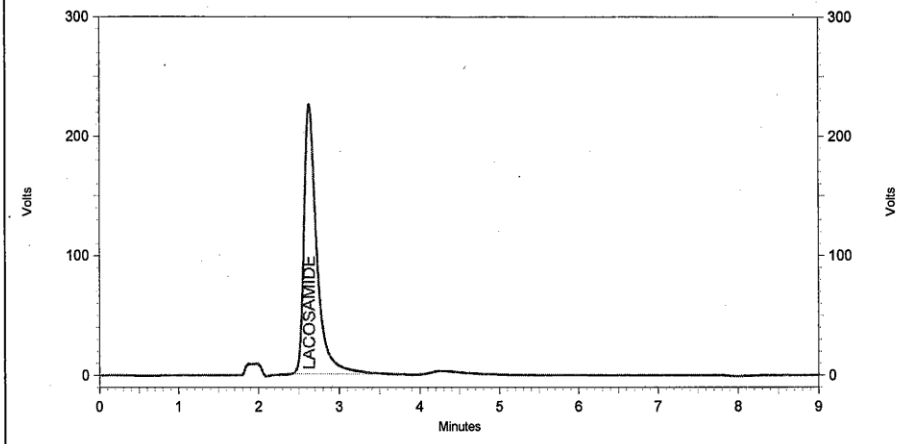
Totals								
		2622059	100.00	231735	100.00			

FIGURE-25

CHROMATOGRAM FOR FIRST RECOVERY ANALYSIS OF FORMULATION (LACOSAM)

Area % Report

Description: RECOVERY SPL-01_LACOSAM-100 (100MCG/ML) + STANDARD (10MCG/ML)
Data File: D:\ChromQuest\PROJECTS\DATA\LACOSAMIDE\FEB 2012\19.02.2012\A023RECOVERY_10%.dat
Method: D:\ChromQuest\Method\LACOSAMIDE FP.met
Acquired: 2/19/2012 6:56:51 AM
Wave Length: 210 nm



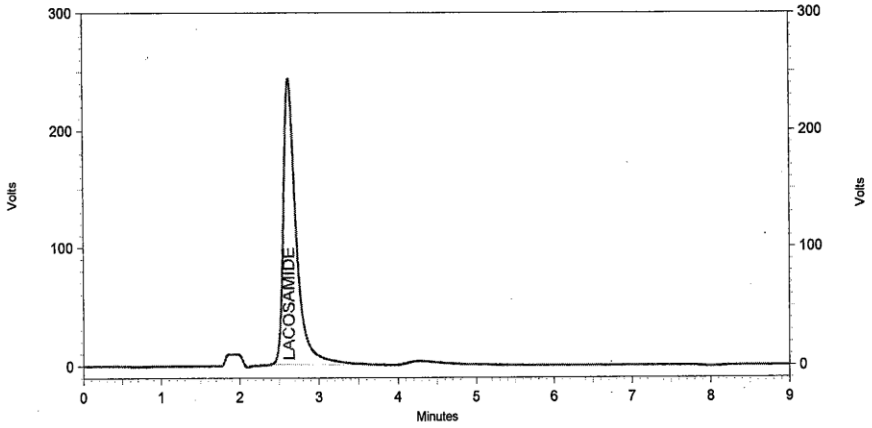
Name	Retention Time	Area	Area %	Height	Height Percent	Asymmetry	Theoretical plates (USP)	Capacity factor
LACOSAMIDE	2.655	2490773	100.00	225225	100.00	1.50	1539	1.7
Totals		2490773	100.00	225225	100.00			

FIGURE-26

CHROMATOGRAM FOR SECOND RECOVERY ANALYSIS OF FORMULATION (LACOSAM)

Area % Report

Description: RECOVERY SPL-02_LACOSAM-100 (100MCG/ML) + STANDARD (20MCG/ML)
Data File: D:\ChromQuest\PROJECTS\DATA\LACOSAMIDE\FEB 2012\19.02.2012\A025RECOVERY_20%.dat
Method: D:\ChromQuest\Method\LACOSAMIDE FP.met
Acquired: 2/19/2012 7:07:18 AM
Wave Length: 210 nm



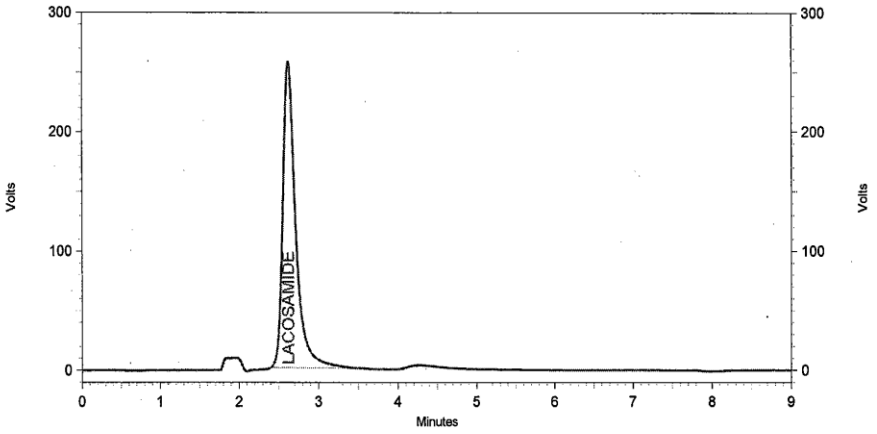
Name	Retention Time	Area	Area %	Height	Height Percent	Asymmetry	Theoretical plates (USP)	Capacity factor
LACOSAMIDE	2.650	2726440	100.00	242497	100.00	1.46	1488	1.7
Totals		2726440	100.00	242497	100.00			

FIGURE-27

CHROMATOGRAM FOR THIRD RECOVERY ANALYSIS OF FORMULATION (LACOSAM)

Area % Report

Description: RECOVERY SPL-03_LACOSAM-100 (100MCG/ML) + STANDARD (30MCG/ML)
Data File: D:\ChromQuest\PROJECTS\DATA\LACOSAMIDE\FEB 2012\19.02.2012\A025RECOVERY_30%.dat
Method: D:\ChromQuest\Method\LACOSAMIDE FP.met
Acquired: 2/19/2012 7:17:43 AM
Wave Length: 210 nm



Name	Retention Time	Area	Area %	Height	Height Percent	Asymmetry	Theoretical plates (USP)	Capacity factor
LACOSAMIDE	2.655	2874684	100.00	256157	100.00	1.48	1441	1.7
Totals		2874684	100.00	256157	100.00			

FIGURE-28

SELECTION OF DETECTION WAVELENGTH BY HPTLC

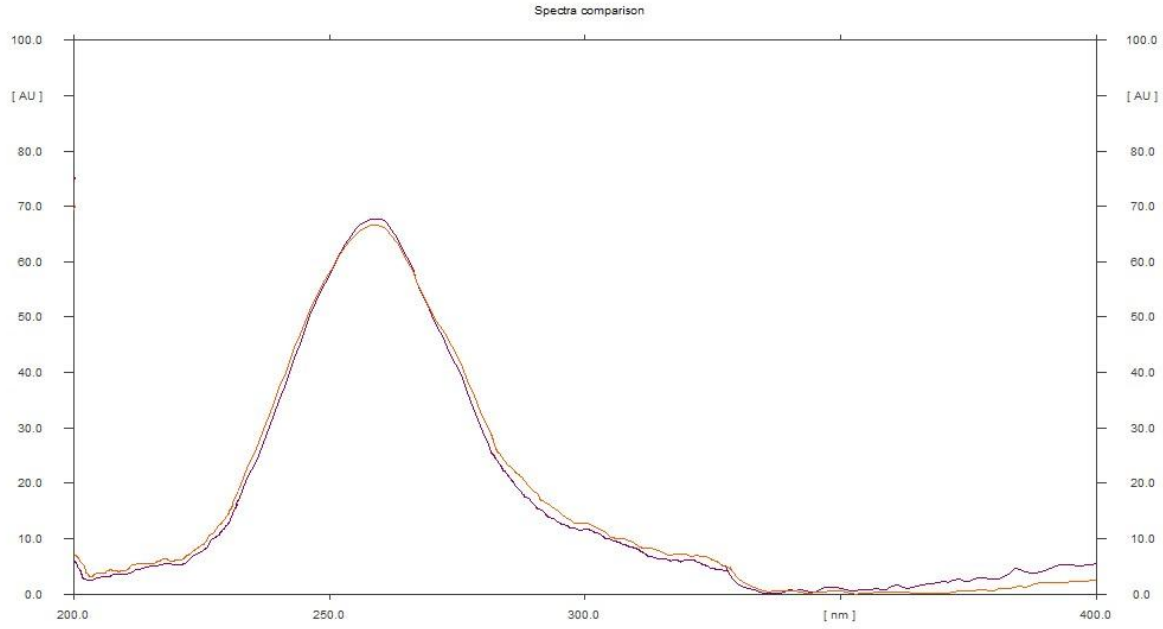


FIGURE-29

LINEARITY CHROMATOGRAM OF LACOSAMIDE

BY HPTLC (1 µg/ mL)

Peak	Start Rf	Start Height	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned Substance
10.64	0.1	0.69	33.6	46.85	0.72	5.3	1059.1	36.33	Lac

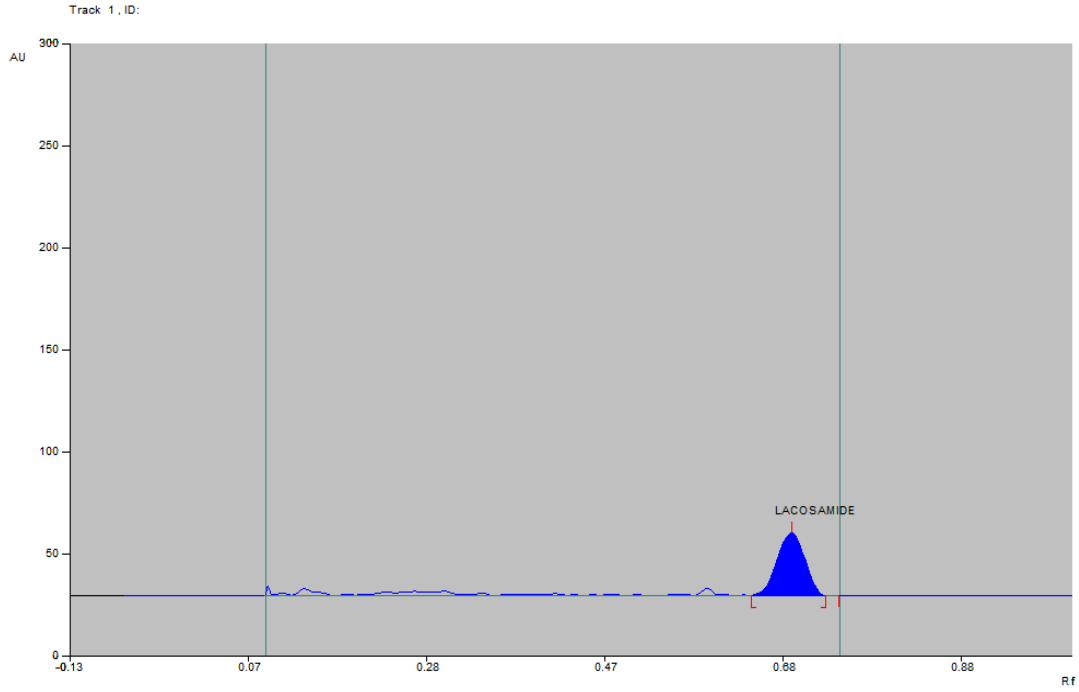


FIGURE-30

LINEARITY CHROMATOGRAM OF LACOSAMIDE

BY HPTLC (2 µg/ mL)

Peak	Start Rf	Start Height	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned Substance
10.61	0.0	0.66	75.4	100.0	0.75	0.69	2146.3	100.00	Lac

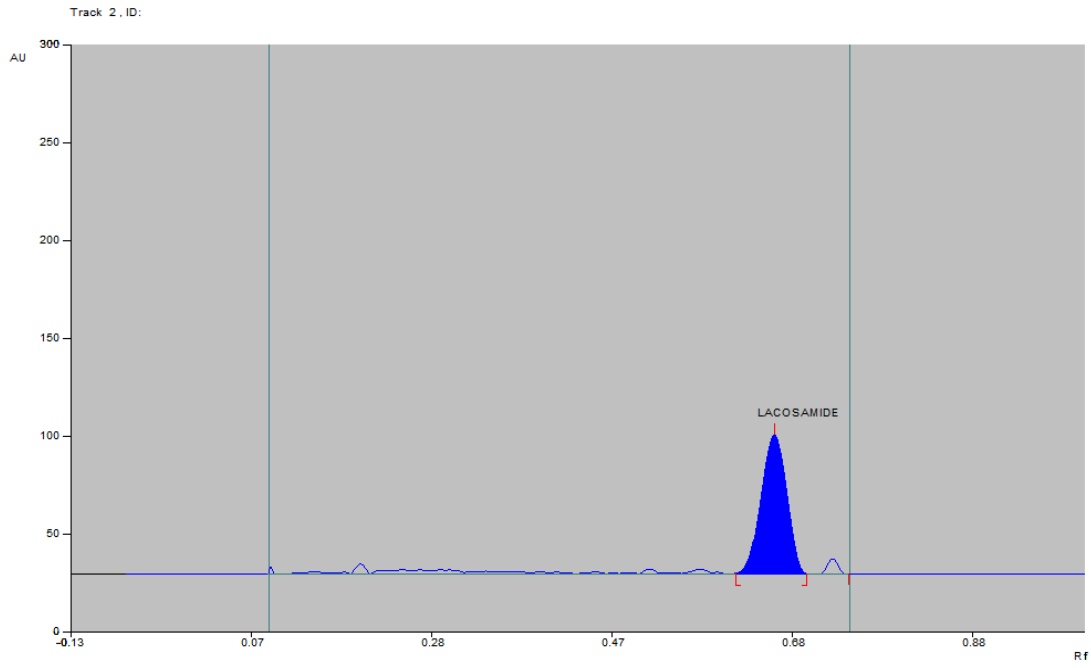


FIGURE-31

LINEARITY CHROMATOGRAM OF LACOSAMIDE

BY HPTLC (3 µg/ mL)

Peak	Start Rf	Start Height	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned Substance
10.60	0.6	0.66	102.1	100.00	0.70	7.6	2970.3	100.00	Lac

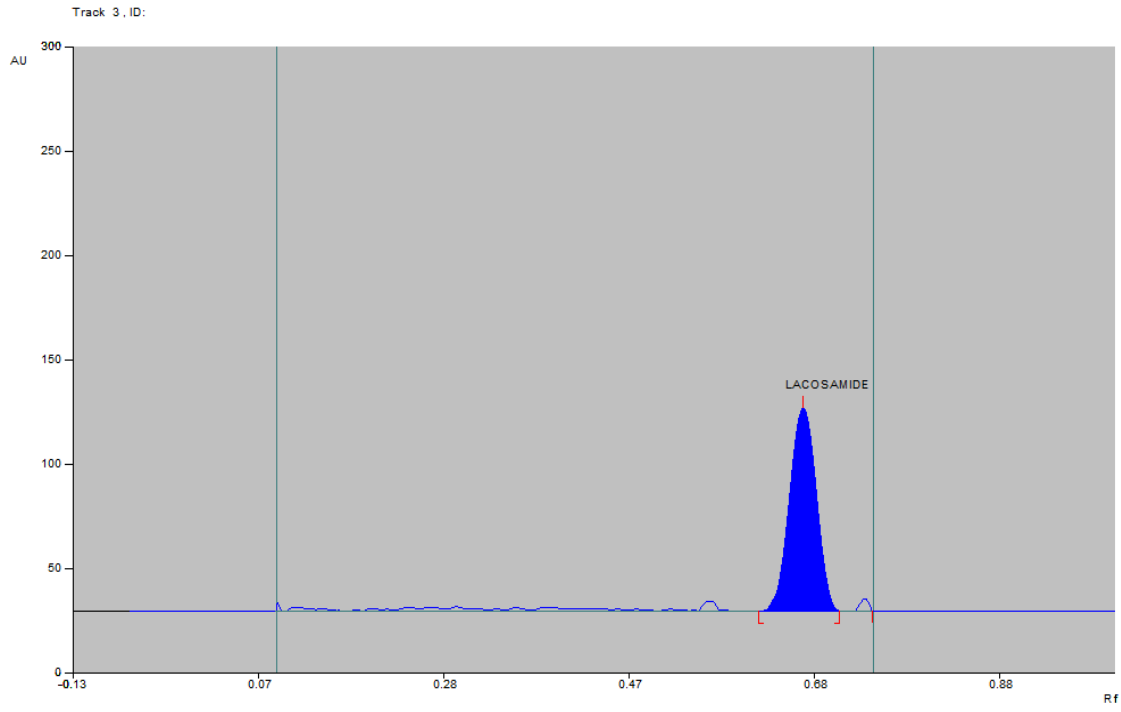


FIGURE-32

LINEARITY CHROMATOGRAM OF LACOSAMIDE

BY HPTLC (4 µg/ mL)

Peak	Start Rf	Start Height	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned Substance
10.62	2.5	0.67	128.9	100.00	0.71	10.8	3864.9	100.00	Lac

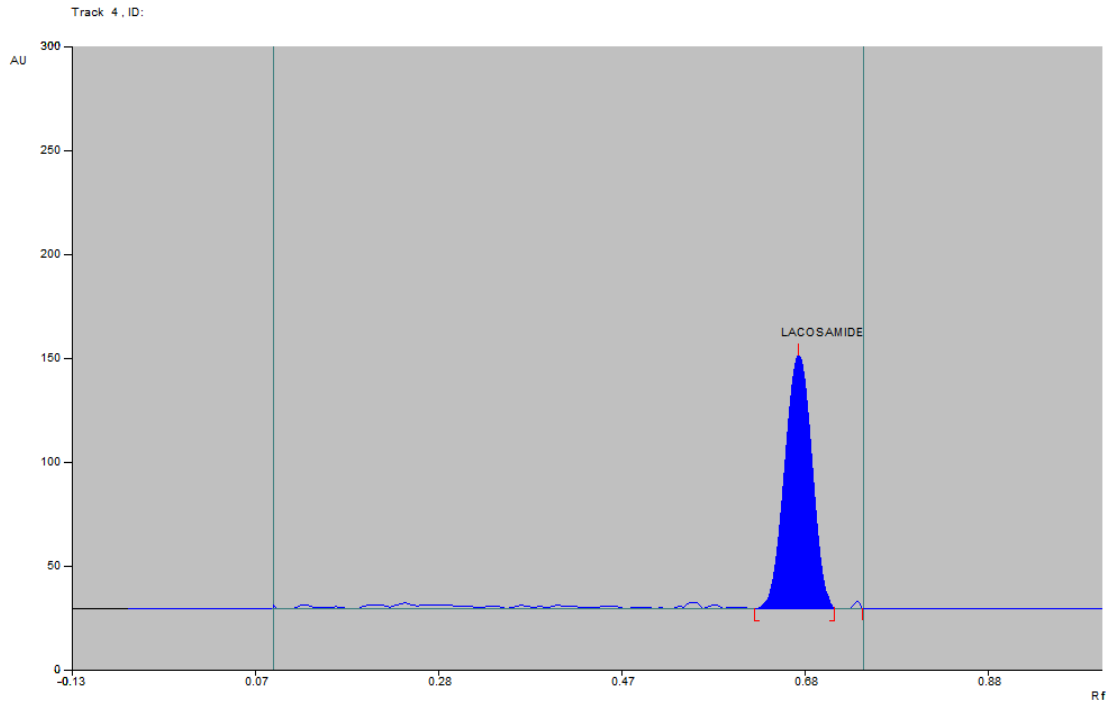


FIGURE-33

LINEARITY CHROMATOGRAM OF LACOSAMIDE

BY HPTLC (5 µg/ mL)

Peak	Start Rf	Start Height	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned Substance
10.61	3.6	0.67	128.9	100.00	0.71	10.8	3864.9	100.00	Lac

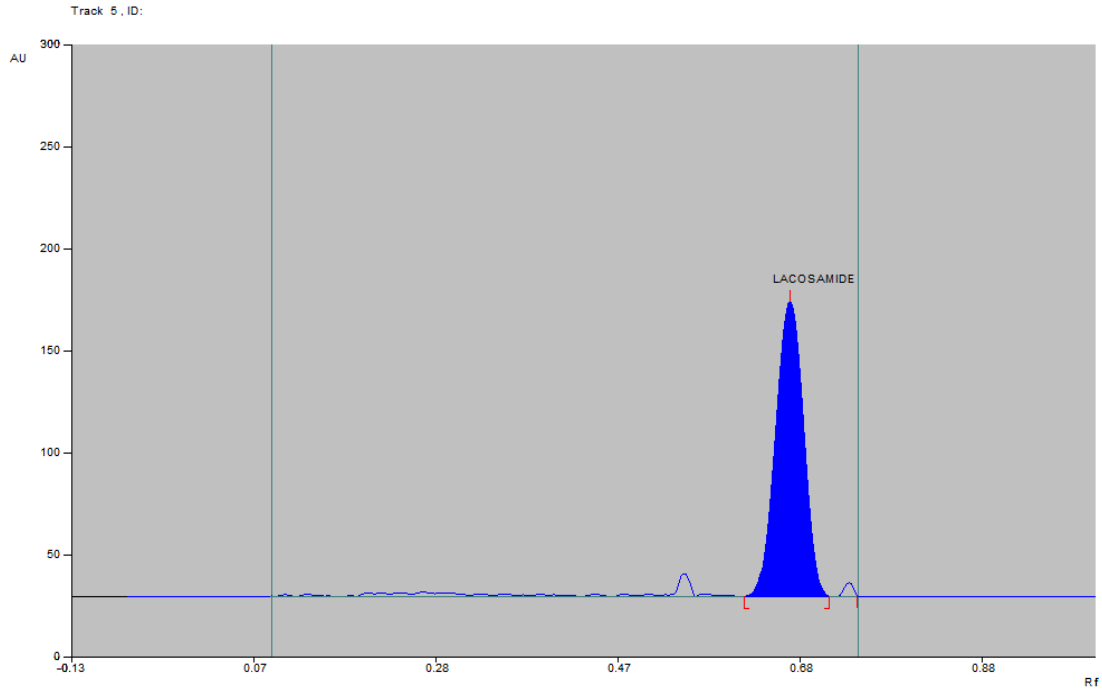


FIGURE-34

LINEARITY CHROMATOGRAM OF LACOSAMIDE

BY HPTLC (6 µg/ mL)

Peak	Start Rf	Start Height	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned Substance
10.60	3.3	0.66	180.6	100.00	0.70	16.5	5669.0	100.00	Lac

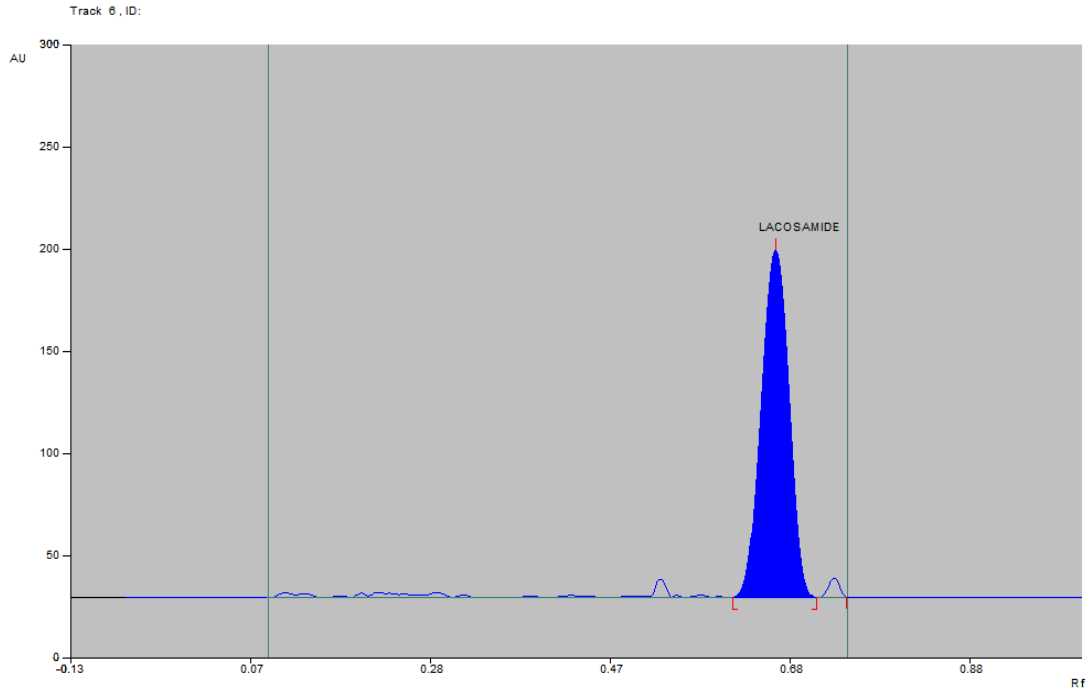


FIGURE-35

CALIBRATION CURVE OF LACOSAMIDE

BY HPTLC

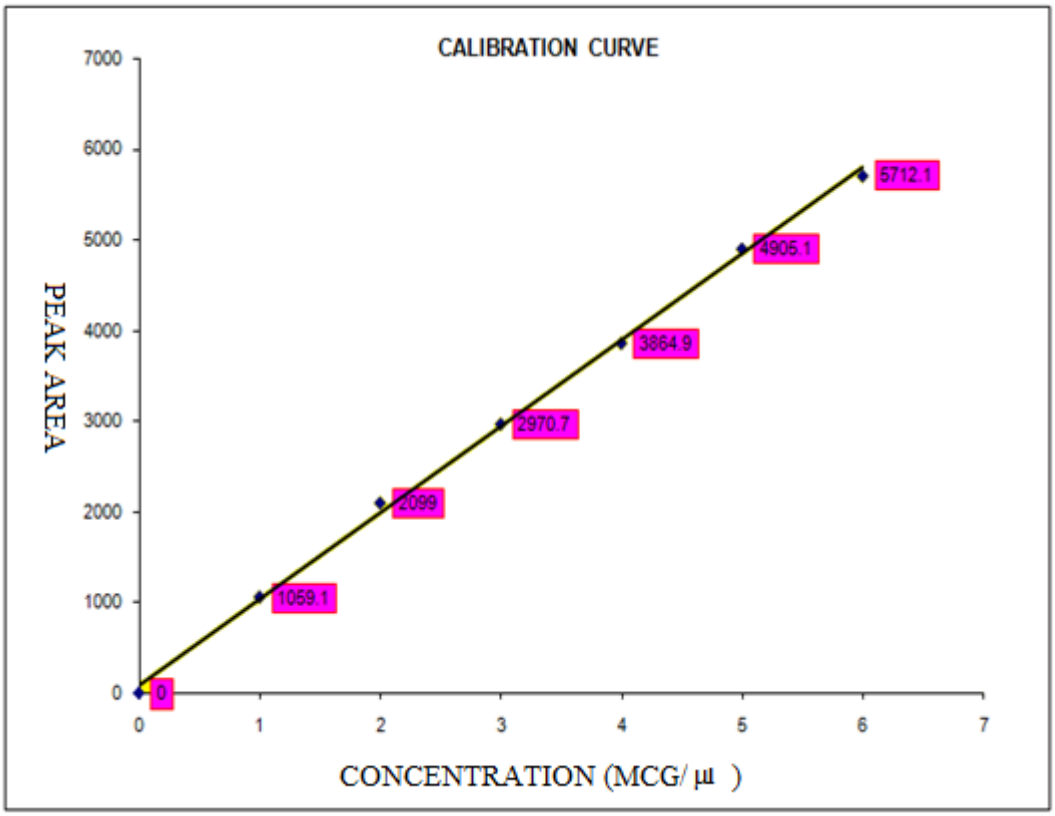


FIGURE-36
CHROMATOGRAM FOR THE ANALYSIS OF LACOSAMIDE FORMULATION (LACOSAM)
REPEATABILITY-1

Peak	Start Rf	Start Height	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned Substance
10.63	0.2	0.67	128.9	100.0	0.70	1.0	2821	100.00	Lac

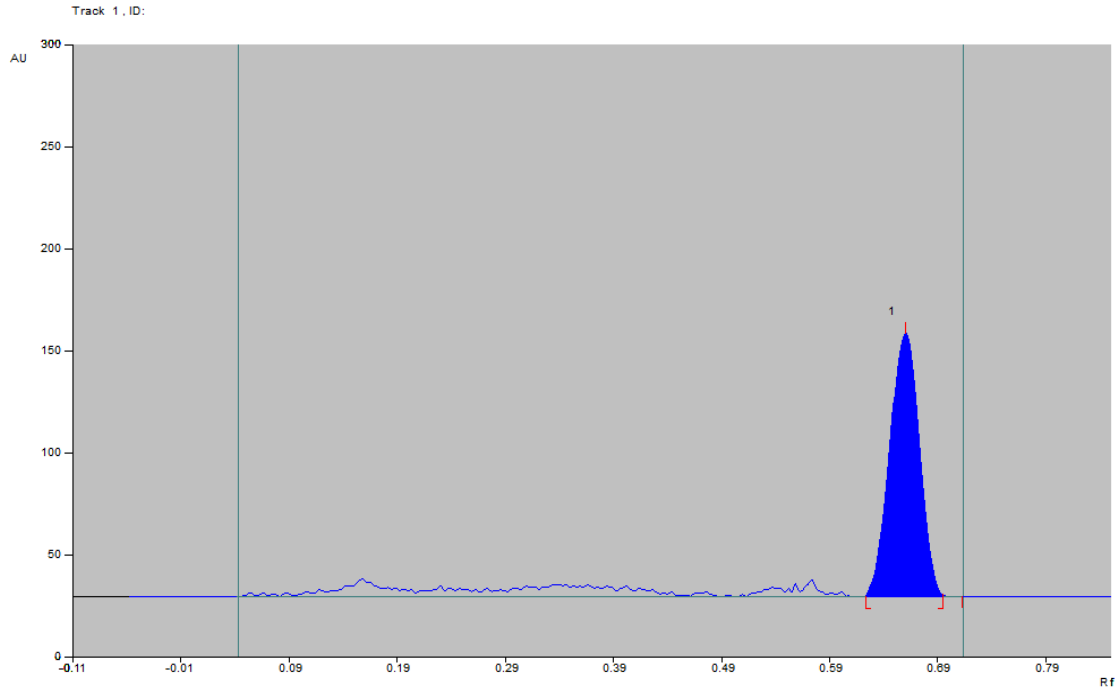


FIGURE-37
CHROMATOGRAM ANALYSIS FOR THE OF LACOSAMIDE FORMULATION (LACOSAM) REPEATABILITY-2

Peak	Start Rf	Start Height	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned Substance
10.61	0.0	0.66	75.4	100.0	0.75	0.69	2970	100.00	Lac

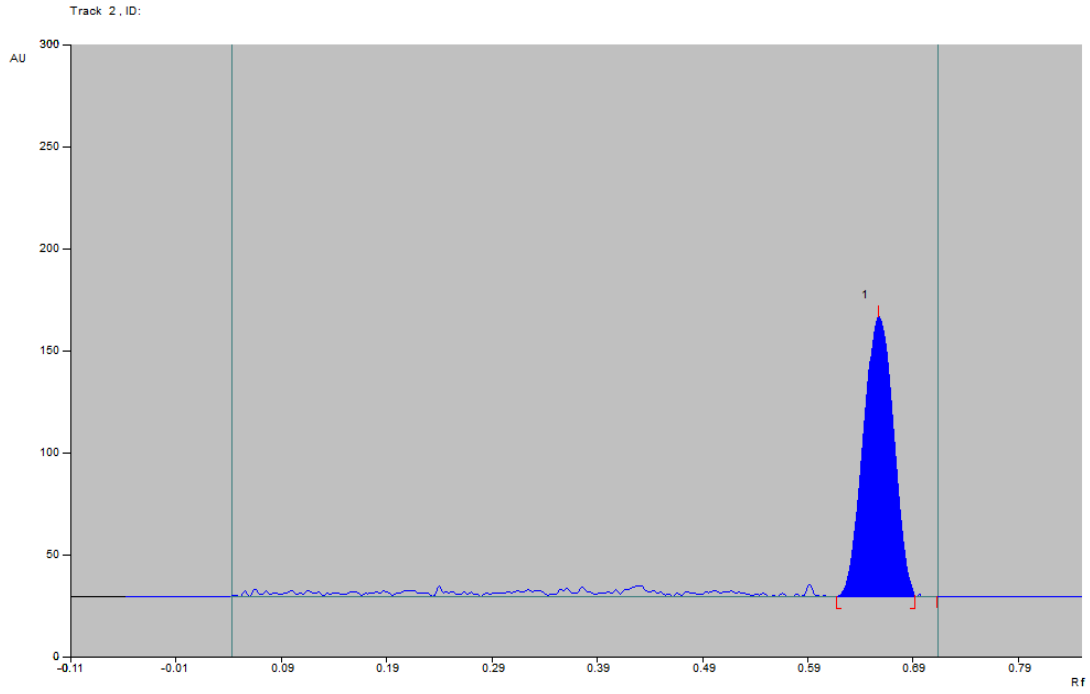


FIGURE-38
CHROMATOGRAM ANALYSIS FOR THE OF LACOSAMIDE FORMULATION (LACOSAM) REPEATABILITY-3

Peak	Start Rf	Start Height	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned Substance
10.62	0.0	0.66	137.1	100.0	0.70	0.6	2906.9	100.00	Lac

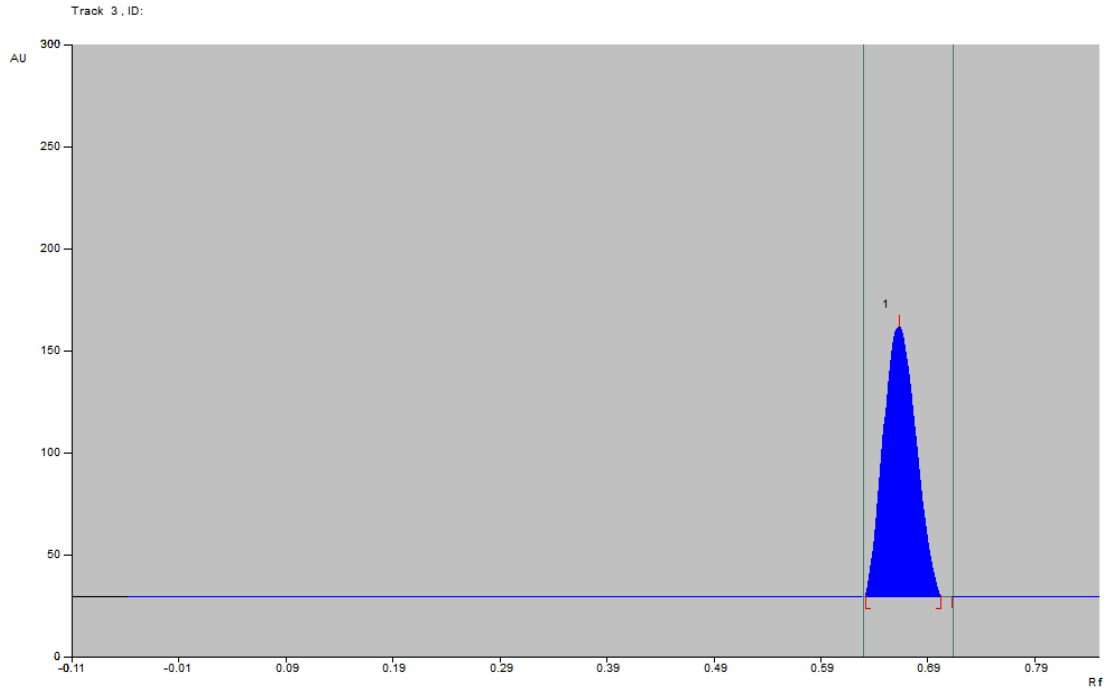


FIGURE-39
CHROMATOGRAM ANALYSIS FOR THE OF LACOSAMIDE FORMULATION (LACOSAM) REPEATABILITY-4

Peak	Start Rf	Start Height	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned Substance
30.67	5.9	0.67	132.3	78.86	0.71	0.4	2959.6	100.00	Lac

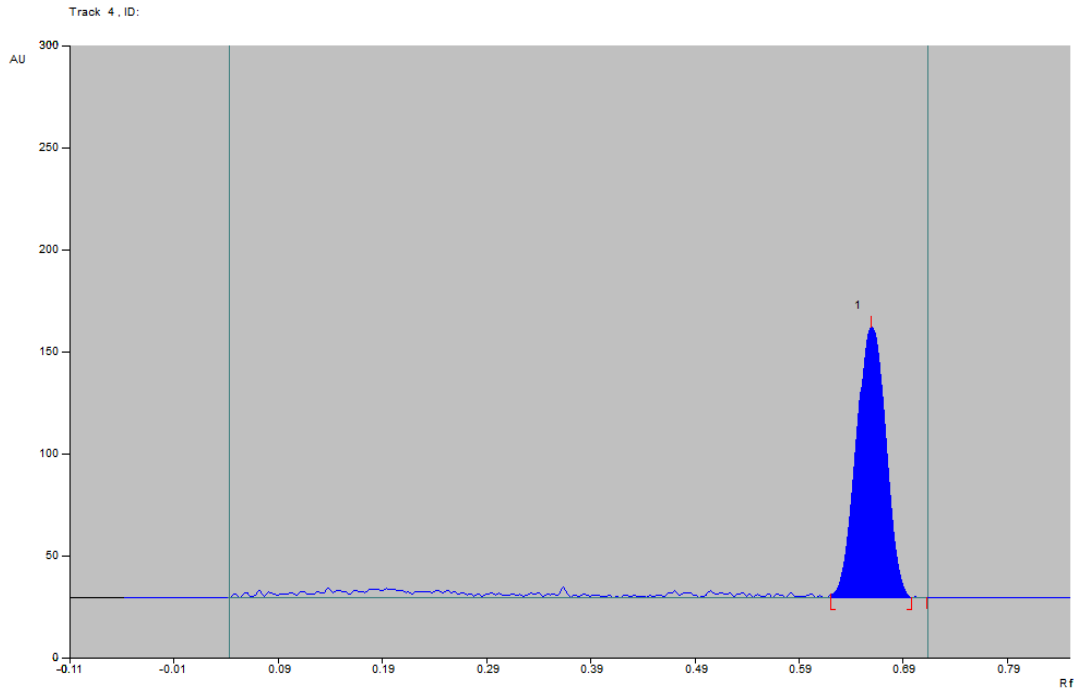


FIGURE-40
CHROMATOGRAM ANALYSIS FOR THE OF LACOSAMIDE FORMULATION (LACOSAM) REPEATABILITY-5

Peak	Start Rf	Start Height	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned Substance
10.63	1.5	0.66	132.7	100.0	0.70	0.0	2945	100.00	Lac

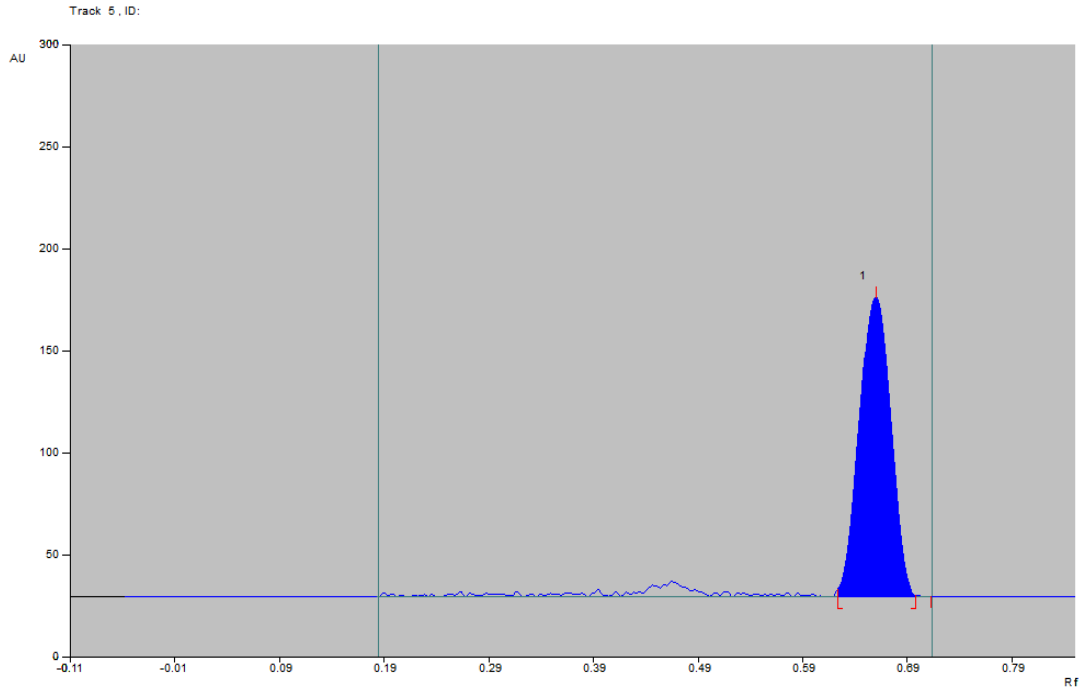


FIGURE-41
CHROMATOGRAM ANALYSIS FOR THE OF LACOSAMIDE FORMULATION (LACOSAM) REPEATABILITY-6

Peak	Start Rf	Start Height	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned Substance
30.67	5.9	0.67	132.3	78.86	0.71	0.4	2959.6	100.00	Lac

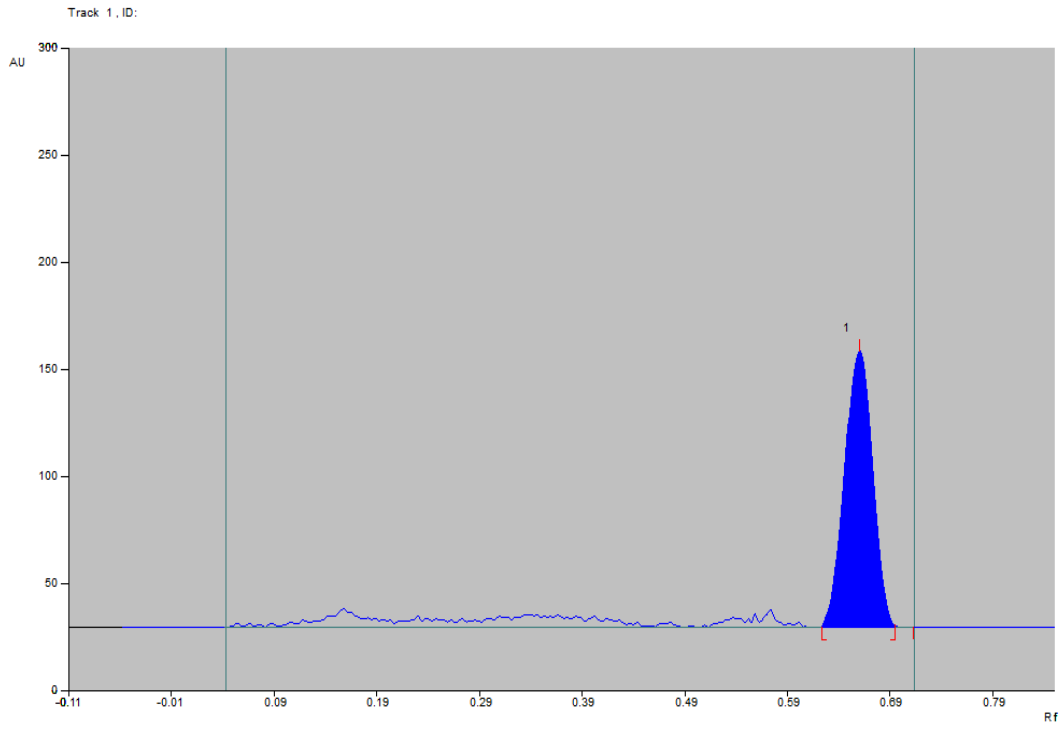


FIGURE-42

CHROMATOGRAM FOR FIRST RECOVERY ANALYSIS OF FORMULATION (LACOSAM) BY HPTLC

Peak	Start Rf	Start Height	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned Substance
10.61	0.0	0.66	75.4	100.0	0.75	0.69	3267	100.00	Lac

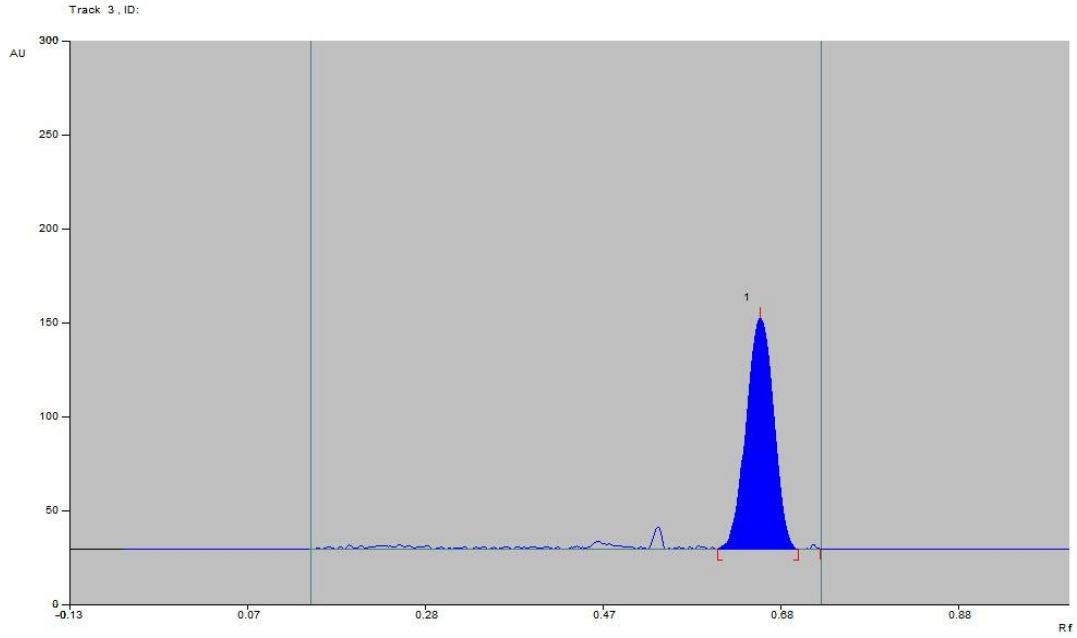


FIGURE-43

CHROMATOGRAM FOR SECOND RECOVERY ANALYSIS OF FORMULATION (LACOSAM) BY HPTLC

Peak	Start Rf	Start Height	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned Substance
10.61	0.0	0.66	75.4	100.0	0.75	0.69	3564	100.00	Lac

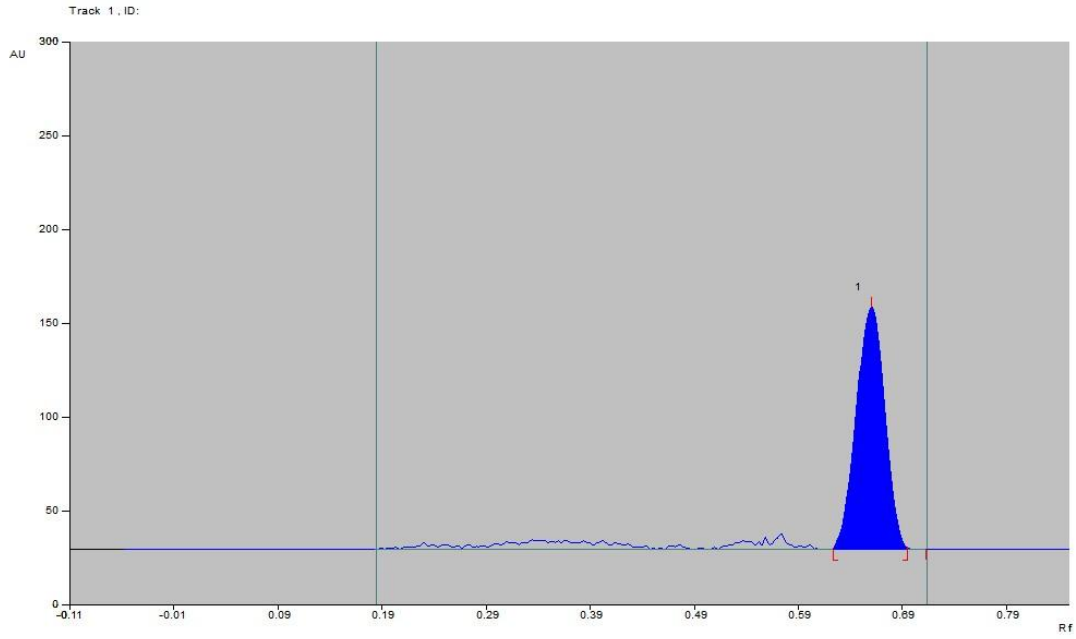
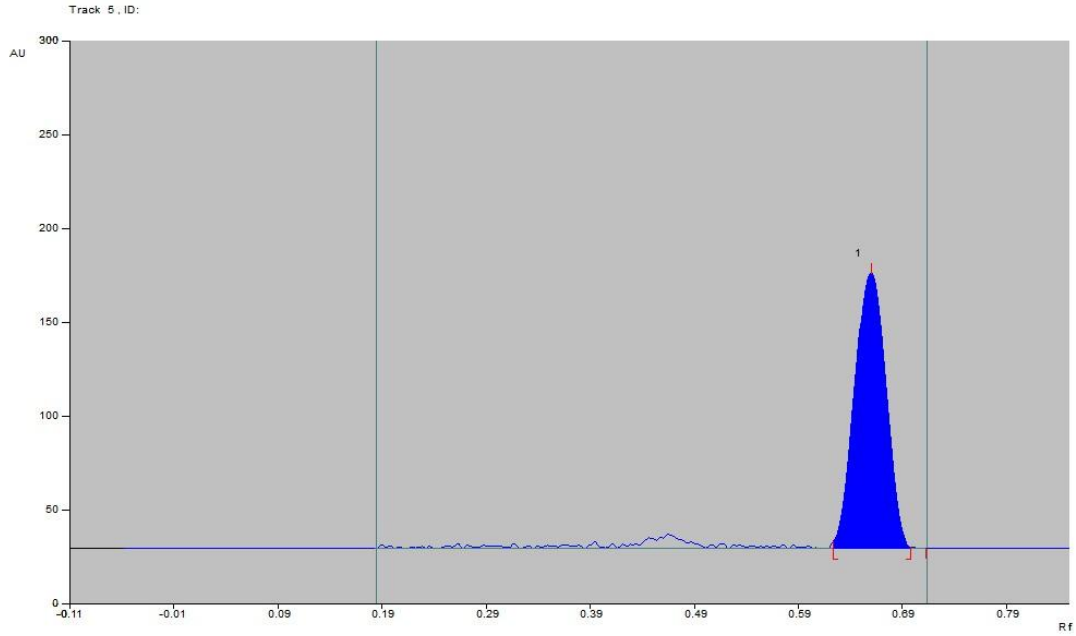


FIGURE-44
CHROMATOGRAM FOR THIRD RECOVERY ANALYSIS OF FORMULATION (LACOSAM) BY HPTLC

Peak	Start Rf	Start Height	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned Substance
10.61	0.0	0.66	75.4	100.0	0.75	0.69	3861	100.00	Lac



TABLES

TABLE 1

SOLUBILITY PROFILE OF LACOSAMIDE IN POLAR AND NON POLAR SOLVENTS

S.NO	SOLVENT	EXTENT OF SOLUBILITY	CONCLUSION
1	Distilled water	10 mg in 400µl	Sparingly soluble
2	Benzene	10 mg in 10 mL	Slightly soluble
3	Methanol	10 mg in 50µl	Freely soluble
4	Dimethyl formamide	10 mg in 50µl	Freely soluble
5	Chloroform	10 mg in 50µl	Freely soluble
6	Ethanol	10 mg in 300µl	Soluble
7	Acetonitrile	10 mg in 50µl	Freely soluble
8	Toluene	10 mg in 10mL	Very slightly soluble
9	Acetone	10 mg in 50µl	Freely soluble
10	Ethyl acetate	10 mg in 50µl	Freely soluble
11	Isopropyl alcohol	10 mg in 50µl	Freely soluble
12	n-butanol	10 mg in 50µl	Freely soluble
13	Diethyl ether	10 mg in 3mL	Slightly soluble
14	Dil. Acetic acid	10 mg in 5mL	Slightly soluble
15	Phosphate buffer pH 8.5	10 mg in 2mL	Slightly soluble
16	0.1M hydrochloric acid	10 mg in 2mL	Slightly soluble
17	Carbon tetra chloride	10 mg in more than 10mL	Practically insoluble
18	Distilled Water	10mg in more than 100mL	Practically insoluble
19	Dimethylsulphoxide	10 mg in 50µl	Freely soluble

TABLE 2

OPTICAL CHARACTERISTICS OF LACOSAMIDE BY FIRST ORDER DERIVATIVE SPECTROPHOTOMETRIC METHOD

S.NO	PARAMETERS	DERIVATIVES
1	λmax (nm)	216.5 nm
2	Beers law limit (µg/mL)	10-50
3	Correlation coefficient (r)	0.999878769
4	Regression equation (y=mx+c)	Y=0.003420429x- 0.000875397
5	Slope (m)	0.003461429
6	Intercept (c)	0.000875397
7	LOD (µg/mL)	0.32951216.53
8	LOQ(µg/mL)	0.998522132
9	Sandell s sensitivity (µg/cm^2 0.001A.U)	0.292400562
10	Standard error of mean	0.000183206

TABLE 3

QUANTIFICATION OF FORMULATION (LACOSAM) BY FIRST ORDER DERIVATIVE SPECTROPHOTOMETRIC METHOD

S. No	Labeled Amount	Amount Found	% Obtained	Average % Found	S.D	% R.S.D	S.E
1	100 mg	97.97 mg	97.97	99.55	1.1795	1.1848	0.0327
2	100 mg	100.7 mg	100.7				
3	100 mg	100.7 mg	100.7				
4	100 mg	98.27 mg	98.27				
5	100 mg	99.93 mg	99.93				
6	100 mg	99.73 mg	99.73				

TABLE 4

INTRADAY PRECISION ANALYSIS OF FORMULATION (LACOSAM) BY FIRST ORDER DERIVATIVE SPECTROPHOTOMETRIC METHOD

Drug	S. No	Labeled amount (mg/tab)	Amount found (mg/tab)	Percentage obtained	Average percentage	S.D	% R.S.D	S.E
Lacosamide	1	100	101.4	101.40	100.93	0.4163	0.4124	0.0462
	2	100	100.8	100.80				
	3	100	100.6	100.60				

*mean of three observation

TABLE 5

INTERDAY PRECISION ANALYSIS OF FORMULATION (LACOSAM) BY FIRST ORDER DERIVATIVE SPECTROPHOTOMETRIC METHOD

Drug	S.No	Labeled amount (mg/tab)	Amount found (mg/tab)	Percentage obtained	Avg %	S.D	% R.S.D	S.E
Lacosamide	1	100	100.9	100.90	100.7	0.2	0.1986	0.0222
	2	100	100.7	100.70				
	3	100	100.5	100.50				

★mean of three observation

TABLE 6

RUGGEDNESS ANALYSIS OF FORMULATION (LACOSAM 100 MG) BY FIRST ORDER DERIVATIVE SPECTROPHOTOMETRIC METHOD

Drug	Condition	Sample No	Labelled Amount (Mg/Tab)	Amount Found (Mg/Tab)	Avg (%) Obtained	S.D	% R.S.D	S.E
Lacosamide	ANALYST 1	1	100	100.70	100.56	0.1250	0.1243	0.0034
		2	100	100.60				
		3	100	100.43				
		4	100	100.50				
		5	100	100.70				
		6	100	100.43				
	ANALYST 2	1	100	100.43	100.36	0.0816	0.0813	0.0022
		2	100	100.33				
		3	100	100.43				
		4	100	100.33				
		5	100	100.23				
		6	100	100.43				

★Mean of six observation

TABLE 7

RUGGEDNESS ANALYSIS OF FORMULATION (LACOSAM 100 MG) BY FIRST ORDER DERIVATIVE SPECTROPHOTOMETRIC METHOD

Drug	Condition	Sample No	Labelled Amount (Mg/Tab)	Amount Found (Mg/Tab)	Avg (%) Obtained	S.D	% R.S.D	S.E
Lacosamide	Instrument 1	1	100	100.60	100.51	0.1124	0.1118	0.0031
		2	100	100.43				
		3	100	100.60				
		4	100	100.50				
		5	100	100.33				
		6	100	100.60				
	Instrument 2	1	100	100.43	100.46	0.0909	0.0905	0.0025
		2	100	100.50				
		3	100	100.33				
		4	100	100.60				
		5	100	100.43				
		6	100	100.50				

TABLE 8

RECOVERY STUDIES FORMULATION (LACOSAM 100 MG) BY DERIVATIVE SPECTROPHOTOMETRIC METHOD

Drug	%	Amt present (µg/mL)	Amt added (µg/mL)	Amt estimated	Amt recovery	% recovery	Avg % recovery	S.D	% R.S.D	S.E
Lacosamide	80	14.92	24	38.80	23.88	99.50	99.81	0.2936	0.2941	0.032
		14.92	24	38.89	23.97	99.87				
		14.92	24	38.94	24.02	100.08				
	100	14.92	30	44.56	29.64	98.80	99.18	1.0356	1.0441	0.115
		14.92	30	44.44	29.52	98.40				
		14.92	30	45.03	30.11	100.36				
	120	14.92	36	50.87	35.95	99.80	99.89	0.1824	0.1826	0.020
		14.92	36	50.96	36.04	100.1				
		14.92	36	50.84	35.92	99.77				

★mean of three observation

TABLE 9

SYSTEM SUITABILITY PARAMETERS FOR THE OPTIMIZED CHROMATOGRAM BY RP - HPLC

Parameters	**Lacosamide**
Retention time	2.690
Tailing factor	1.26
Asymmetrical factor	1.50
Theoretical plates	1536
Capacity factor	1.7

TABLE 10

OPTICAL CHARACTERISTICS OF LACOSAMIDE BY RP – HPLC

Parameters	**Lacosamide**
λ_{max} (nm)	210
Beers law limit (µg/mL)	70 -130
Correlation coefficient ®	0.9997
Regression equation (y=mx+c)	y= 23347.4513x + 126139.6429
Slope (m)	23347.4513
Intercept (C)	126139.6429
LOD (µg/mL)	0.20919
LOQ (µg/mL)	0.633912
Standard Error	15287.54312

TABLE - 11

ANALYSIS OF TABLET FORMULATION (LACOSAM) BY RP-HPLC

Drug	Sample ID.	Labeled amount (mg/tab)	Amount found (mg/tab)*	Percentage Obtained*	Average (%)	S.D	% R.S.D.	S.E.
LAC	Low level 1	100	99.208	99.20	99.47	0.3760	0.3768	0.0046
	Low level 2	100	100.204	100.20				
	Low level 3	100	99.023	99.02				
	Middle level 4	100	99.423	99.42				
	Middle level 5	100	99.550	99.55				
	Middle level 6	100	99.381	99.38				
	High level 7	100	99.069	99.06				
	High level 8	100	99.705	99.70				
	High level 9	100	99.755	99.75				

TABLE 12

RECOVERY STUDY DATA OF 100 % PREANALYZED FORMULATION (LACOSAM) BY RP-HPLC

Drug	Amount present (mcg/mL)	Amount added (mcg/mL)	Amount estimated (mcg/mL)	Amount recovered (mcg/mL)	% Recovery	S.D	% R.S.D	S.E.
LAC	100	10	109.521	10.041	100.41	0.4450	0.4455	0.0494
	100	20	119.398	19.918	99.59			
	100	30	129.391	29.911	99.70			

TABLE 13

OPTICAL CHARACTERISTIC OF LACOSAMIDE BY HPTLC METHOD

S.NO	PARAMETERS	VALUES
1	λmax (nm)	257 nm
2	Beers law limit (ng/mL)	1000-6000
3	Correlation coefficient (r)	0.9992
4	Regression equation (y=mx+c)	Y=954.79X+97.63
5	Slope (m)	954.79
6	Intercept (c)	97.63
7	LOD (µg/mL)	0.0540
8	LOD (µg/mL)	0.1638
9	Sandell s sensitivity (µg/cm^2 0.001A.U)	1.047
10	Standard error of mean	2.3559

TABLE 14

QUANTIFICATION OF FORMULATION (LACOSAM) BY HPTLC METHOD

S.No	Labeled Amount	Amount Found	Percentage Obtained	Avg %	S.D	% R.S.D	S.E
1	50 mg	49.56 mg	99.13				
2	50 mg	49.23 mg	99.47				
3	50 mg	50.19 mg	100.38				
4	50mg	50.13 mg	100.26	99.13	1.1737	1.1840	0.03260
5	50mg	48.70 mg	97.40				
6	50mg	49.08 mg	98.16				

★mean of six observation

TABLE 15
INTRADAY PRECISION ANALYSIS OF FORMULATION (LACOSAM) BY HPTLC METHOD

Drug	S.No	Labeled amount (mg/tab)	Amount found (mg/tab)	Percentage obtained	Average percentage	S.D	% R.S.D	S.E
Lac	1	50	49.08	98.16	98.30	0.3442	0.3502	0.0382
	2	50	49.03	98.06				
	3	50	49.39	98.70				

★mean of three observation

TABLE 16

INTERDAY PRECISION ANALYSIS OF FORMULATION

(LACOSAM) BY HPTLC METHOD

Drug	S.No	Labeled amount (mg/tab)	Amount found (mg/tab)	Percentage obtained	Average percentage	S.D	% R.S.D	S.E
Lacosamide	1	50	49.39	98.78	99.54	1.4153	1.4218	0.1572
	2	50	50.59	101.18				
	3	50	49.34	98.68				

★mean of three observation

TABLE 17

RECOVERY STUDIES FORMULATION (LACOSAM 50 MG) BY HPTLC METHOD

Drug	%	Amount present (µg/mL)	Amount added (µg/mL)	Amt found	Amt recovery	% recovery	Avg % recovery	S.D	% R.S.D	S.E
Lacosamide	80	3.015	2.4	5.3932	2.3782	99.09				
	100	3.015	3.0	6.0161	3.001	100.00	100.36	1.687	1.679	0.187
	120	3.015	3.6	6.700	3.685	102.36				

★mean of three observation

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